UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Agrícola y Bromatología

PEPTIDE-BASED STRATEGIES FOR PREVENTION AND TREATMENT OF EGG ALLERGY

ESTRATEGIAS BASADAS EN PÉPTIDOS PARA LA PREVENCIÓN Y EL TRATAMIENTO DE LA ALERGIA AL HUEVO

DANIEL LOZANO OJALVO

INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC-UAM)

Madrid 2016

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Agrícola y Bromatología

PEPTIDE-BASED STRATEGIES FOR PREVENTION AND TREATMENT OF EGG ALLERGY

ESTRATEGIAS BASADAS EN PÉPTIDOS PARA LA PREVENCIÓN Y EL TRATAMIENTO DE LA ALERGIA AL HUEVO

Memoria presentada por:

Daniel Lozano Ojalvo

Para optar al grado de

DOCTOR

CON MENCIÓN DE "DOCTORADO INTERNACIONAL"

Instituto de Investigación en Ciencias de la Alimentación

Trabajo realizado bajo la dirección de:

Dra. Rosina López-Alonso Fandiño

Dra. Elena Molina Hernández

ROSINA LÓPEZ-FANDIÑO, PROFESORA DE INVESTIGACIÓN, Y ELENA MOLINA, CIENTÍFICA TITULAR, AMBAS CON DESTINO EN EL INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN DEL CSIC

INFORMAN:

Que el trabajo titulado "PEPTIDE-BASED STRATEGIES FOR PREVENTION AND TREATMENT OF EGG ALLERGY / ESTRATEGIAS BASADAS EN PÉPTIDOS PARA LA PREVENCIÓN Y EL TRATAMIENTO DE LA ALERGIA AL HUEVO" constituye la memoria que presenta el Licenciado en Veterinaria D. DANIEL LOZANO OJALVO para optar al grado de Doctor con Mención de Doctorado Internacional. Esta tesis doctoral se ha realizado bajo la dirección de las Dras. Rosina López-Fandiño y Elena Molina, en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM).

Y para que conste firmamos el presente informe a 28 de Noviembre de 2016.

Fdo.: Rosina López-Alonso Fandiño

Fdo.: Elena Molina Hernández

V°B° Tutora:

Fdo.: María de los Ángeles Martín Cabrejas

C/ Nicolás Cabrera, 9.
Campus de Cantoblanco
UAM
28049 Madrid

“Caminando en línea recta no puede uno llegar muy lejos”

“Droit devant soi on ne peut pas aller bien loin”

(Le petit prince, Antoine de Saint-Exupéry, 1943)

CONTENTS

ABSTRACT

Egg allergy is among the most common food allergies in European children below the age of three, with a prevalence ranging between 0.2 and 2%. The management of this allergic disease is limited to strict avoidance of the offending food, although total evasion is difficult from a practical standpoint since egg and egg-derived products are ubiquitous ingredients. In this regard, oral immunotherapy (OIT) using whole egg white (EW) appears as an effective treatment option for egg allergy, with a success rate between 50 and 90%. However, a major drawback of OIT using intact egg allergens is the high risk of severe side effects. This obstacle has prompted the investigation of strategies aimed at reducing the allergenicity of the egg-derived preparations used for IT. Among the most promising approaches currently studied, stands the possibility of using allergen-derived immunomodulating peptides to induce oral tolerance towards offending food proteins or even to prevent allergic sensitization. In this respect, hydrolysis of egg white proteins appears as an attractive and safe alternative for a reproducible and standardized production of immune-active peptides with low associated costs.

Given this scenario, the aim of this thesis was the development of enzymatic hydrolysates of egg white proteins with hypoallergenic and immunomodulating properties that could be used in the prevention and treatment of egg allergy. With this purpose, enzymatic hydrolysates of ovalbumin (OVA), lysozyme, ovomucoid and EW were prepared using pepsin, Neutrase and alcalase. These hydrolysates were fractionated, characterized by chromatographic and mass spectrometric techniques, and their potential residual allergenicity assessed by *in vitro* and *in vivo* assays. All the hydrolysates produced with alcalase presented a very low binding to IgE from egg allergic patients and that of OVA with pepsin also showed a decreased IgE binding capacity. The hypoallergenicity of these hydrolysates was confirmed by the evaluation of the local allergic responses induced after their administration to mice passively sensitized to egg white proteins.

The immunomodulating effect of the hydrolysates was first evaluated in murine spleen and mesenteric lymph node cells stimulated with T or B cell mitogens. The results suggested that the hydrolysates produced with alcalase decreased the production of Th2-biased cytokines. In addition, these hydrolysates, together with that of OVA with pepsin, inhibited IgG1 class switching, which is also part of the IL-4 mediated Th2 response, and reduced the release of reactive oxygen species (ROS). We next assessed their ability to hinder cytokine and IgE production by Th2-skewed human peripheral blood mononuclear cells (PBMCs), as well as the release of pro-inflammatory factors and generation of ROS from Th1-stimulated peripheral blood leukocytes (PBLs). Data showed that the hydrolysates with alcalase and that of OVA with pepsin helped to re-establish the Th1/Th2 balance in Th2-biased PBMCs, while they also inhibited the release of pro-inflammatory mediators and reduced oxidative stress in PBLs treated with inflammatory stimuli. Together, these cellular models allowed us to select hydrolysates with the ability to down-modulate Th2 cytokine and IgE secretion and to attenuate inflammatory responses.

Based on these previous screening tests, we explored the possibility that the selected hypoallergenic and immunostimulating hydrolysates could modulate T cell cytokine responses to egg allergens *ex vivo*, using splenocytes from BALB/c mice sensitized to individual egg proteins or to their mixtures in different proportions. The results obtained revealed that OVA hydrolysed with alcalase and pepsin could be regarded as two good candidates for peptide-based IT on the grounds of their ability to reduce Th2 cytokines induced by egg allergens in the spleen cell cultures. In addition, the hydrolysate of OVA with pepsin also enhanced Th1-related responses.

In the last part of this thesis, we evaluated the sensitizing and eliciting capacities of the selected hydrolysates, as well as their preventive and therapeutic ability, using a mouse model of egg allergy. Results revealed that the hydrolysate of OVA with pepsin was weakly immunogenic and lacked sensitizing and eliciting capacity. For its part, *in vivo* testing of OVA hydrolysed with alcalase revealed immunogenic and antigenic capacities not previously detected

by *in vitro* methods. OVA hydrolysed with pepsin offered preventive and therapeutic protection against allergy to EW, exerting a marked reduction of Th2 and Th1 systemic responses, through the induction of regulatory T cells and the upregulation of TGF-β, IL-10 and IL-17 in intestinal tissues. This hydrolysate was more effective than intact OVA in desensitizing mice by virtue of its lower allergenicity and its immunomodulating capacity.

In general terms, the screening strategy followed in this work proved useful to select a hydrolysate that combines tolerance induction with the absence of undesired effects of IgE-crosslinking and inflammatory cell activation. The results presented in this thesis show that OVA hydrolysed with pepsin is hypoallergenic and contains immunomodulating peptides which lead to the prevention of egg allergy development and the promotion of long term desensitization to egg white proteins, as well as to linked-epitope suppression.

RESUMEN

La alergia al huevo es una de las alergias alimentarias más comunes entre los niños europeos menores de tres años, con una prevalencia que oscila entre el 0,2 y el 2 %. El tratamiento de esta patología se limita a evitar el consumo de los alimentos ofensivos, aunque su evasión completa es complicada desde un punto de vista práctico ya que el huevo y sus productos derivados son ingredientes presentes en un amplio número de preparaciones culinarias. En este sentido, la inmunoterapia oral (OIT) utilizando clara de huevo completa (EW) se presenta como una de las mejores opciones para el tratamiento de la alergia al huevo, con una tasa de éxito que varía entre el 50 y el 90%. Sin embargo, el mayor problema que presenta la OIT con el alérgeno intacto es el alto riesgo de aparición de efectos adversos. Este obstáculo ha promulgado la investigación de estrategias dirigidas a la reducción de la alergenicidad de las preparaciones utilizadas en la OIT. Entre los procedimientos más prometedores, la posibilidad de utilizar péptidos inmunomodulantes derivados de las proteínas alergénicas aparece como una de las mejores opciones para inducir tolerancia oral a los alimentos ofensivos o incluso para prevenir la sensibilización alérgica. En este aspecto, la hidrólisis de las proteínas de clara de huevo se presenta como una alternativa atractiva y segura para la producción reproducible y estandarizada, con un bajo coste asociado, de péptidos inmunológicamente activos.

Dado este escenario, el objetivo de esta tesis doctoral fue el desarrollo de hidrolizados enzimáticos a partir de proteínas de clara de huevo con propiedades hipoalergénicas e inmunomodulantes que puedan ser utilizados en la prevención y el tratamiento de la alergia al huevo. Con este propósito se prepararon hidrolizados de ovalbúmina (OVA), lisozima, ovomucoide y EW utilizando pepsina, Neutrasa y alcalasa. Estos hidrolizados fueron fraccionados y caracterizados por técnicas cromatográficas y de espectrometría de masas. Además, su alergenicidad residual fue estudiada mediante ensayos *in vitro* e *in vivo.* Todos los hidrolizados producidos con alcalasa, presentaron una baja unión a la IgE de pacientes alérgicos al huevo, además, el hidrolizado de OVA con pepsina también mostró una reducida capacidad

de unión a IgE. La hipoalergenicidad de estos hidrolizados fue confirmada mediante la evaluación de la respuesta alérgica local tras su administración a ratones pasivamente sensibilizados a las proteínas de la clara de huevo.

El efecto inmunomodulante de los hidrolizados fue, en primer lugar, evaluado en células de ratón procedentes del bazo y de los nódulos linfáticos mesentéricos, estimuladas con mitógenos de células T o B. Los resultados obtenidos sugirieron que los hidrolizados producidos con alcalasa eran capaces de disminuir la producción de citoquinas del tipo Th2. Además, estos hidrolizados, junto al de OVA con pepsina, inhibieron la producción de anticuerpos IgG1, los cuales forman parte de la respuesta Th2 mediada por IL-4, y redujeron la liberación de especies reactivas de oxígeno (ROS). A continuación, evaluamos su habilidad para regular la producción de citoquinas e IgE en un modelo de células mononucleares humanas de sangre periférica (PBMCs) polarizadas hacia una respuesta Th2, así como la liberación de factores pro-inflamatorios y la generación de ROS en leucocitos humanos de sangre periférica (PBLs) inducidos hacia una respuesta del tipo Th1. Los datos mostraron que los hidrolizados producidos con alcalasa, así como el de OVA con pepsina, ayudaban a restablecer el balance Th1/Th2 en PBMCs polarizadas hacia Th2, mientras que también fueron capaces de inhibir la liberación de mediadores pro-inflamatorios y de reducir el estrés oxidativos en PBLs tratados con estímulos inflamatorios. En conjunto, estos modelos celulares nos permitieron seleccionar hidrolizados con habilidad para reducir la secreción de IgE y de citoquinas Th2, así como para atenuar las respuestas inflamatorias.

Basándonos en los métodos de evaluación previos, exploramos la posibilidad de que los hidrolizados hipoalergénicos e inmunomodulantes seleccionados pudiesen regular *ex vivo* la producción de citoquinas de células T inducidas en respuesta a los alérgenos del huevo, utilizando esplenocitos procedentes de ratones BALB/c sensibilizados a las proteínas de la clara de huevo, individualmente o a su mezcla en diferentes proporciones. Los resultados obtenidos revelaron que los hidrolizados de OVA con alcalasa y pepsina eran posibles candidatos para ser utilizados en la IT basada en péptido, ya que poseían la habilidad de reducir la producción de

citoquinas Th2 inducida por los alérgenos del huevo en los cultivos de células esplénicas. Además el hidrolizado de OVA con pepsina mostró también una mejora de las respuestas del tipo Th1.

En la última parte de esta tesis, evaluamos la capacidad sensibilizante de los hidrolizados seleccionados y su aptitud para inducir reacciones anafilácticas *in vivo*, así como su habilidad preventiva y curativa en un modelo múrido de alergia al huevo. Los resultados mostraron que el hidrolizado de OVA con pepsin era muy poco inmunogénico y no poseía capacidad ni para sensibilizar a los ratones ni para inducir respuestas anafilácticas en los animales sensibilizados. Por su parte, la evaluación *in vivo* del hidrolizado de OVA con alcalasa mostró que éste presentaba características inmunogénicas y antigénicas que no habían sido previamente detectadas en los modelos *in vitro*. La OVA hidrolizada con pepsina ofreció una protección a la alergia al huevo, tanto preventiva como terapéutica. La administración oral de este hidrolizado mostró una marcada reducción en las respuestas sistémicas del tipo Th2 y Th1 a través de la inducción de células T reguladoras y del incremento de TGF-β, IL-10 and IL-17 en los tejidos intestinales. Además, la hidrólisis de OVA con pepsina fue más efectiva que el uso de la proteína intacta en la desensibilización de los ratones dada su menor alergenicidad y su capacidad inmunomodulante.

En términos generales, la estrategia de evaluación seguida en este trabajo mostró ser útil en la selección de un hidrolizado que es capaz de combinar la inducción a la tolerancia con la ausencia de efectos indeseados relacionados con el entrecruzamiento de las moléculas de IgE y la activación celular. Los resultados presentados en esta tesis doctoral mostraron que la OVA hidrolizada con pepsina es hipoalergénica y contiene péptidos inmunomodulantes que permiten la prevención de la alergia al huevo y proporciona una desensibilización mantenida en el tiempo a las proteínas de la clara del huevo basada en la supresión de epítopos.

ABBREVIATIONS

ACN: acetonitrile

AE-OP: peptide fractions of the hydrolysate of ovalbumin with pepsin separated by anion exchange FPLC

B reg: regulatory B cells

CFSE: carboxyfluorescein succinimidyl ester

Cox-2: cyclooxygenase-2

CT: cholera toxin

DCs: dendritic cells

DCFH-DA: dichloro-dihydro-fluorescein diacetate

DHB: 2.5-dihydroxybenzoic acid matrix

DTT: dithiothreitol

EA: hydrolysate of whole egg white with alcalase

EDTA: ethylenediaminetetraacetic acid

EN: hydrolysate of whole egg white with Neutrase

EP: hydrolysate of whole egg white with pepsin

EW: whole egg white

EW+CT: administration of whole egg white plus cholera toxin

FBS: Fetal bovine serum

FcεRI: high-affinity IgE receptor type I

FcεRII: low-affinity IgE receptor type II (also called CD23)

GALT: gut associated immune system

HLA: Human leukocyte antigen

HLA-DR: Human Leukocyte Antigen - antigen D Related

i.p.: intraperitoneal

IECs: intestinal epithelial cells

IκB*α*: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

IKK: nuclear factor of kappa kinase

ILC2: type 2 innate lymphoid cells

iNOS: inducible nitric oxide synthase

IT: immunotherapy

LA: hydrolysate of lysozyme with alcalase

LN: hydrolysate of lysozyme with Neutrase

LP: hydrolysate of lysozyme with pepsin

LPS: lipopolysaccharide

LYS: lysozyme

MA: hydrolysate of ovomucoid with alcalase

MAPKs: mitogen-activated protein kinases

MHC: major histocompatibility complex

MHC-II: major histocompatibility complex class II

MLN: mesenteric lymph nodes

mMCP-1: mouse mast cell protease-1

MN: hydrolysate of ovomucoid with Neutrase

MP: hydrolysate of ovomucoid with pepsin

MTT: 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazoliumbromide

Muc2: mucin type 2

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO: nitric oxide

OA: hydrolysate of ovalbumin with alcalase

OA+CT: administration of ovalbumin hydrolysed with alcalase plus cholera toxin

OIT: oral immunotherapy

OM: ovomucoid

ON: hydrolysate of ovalbumin with Neutrase

OP: hydrolysate of ovalbumin with pepsin

OP+CT: administration of ovalbumin hydrolysed with pepsin plus cholera toxin

OVA: ovalbumin

PBLs: peripheral blood leukocytes

PBMCs: peripheral blood mononuclear cells

PCA: passive cutaneous anaphylaxis

PGE2: prostaglandin E2

PBS: phosphate buffered saline

PP: Peyer's patches

RMCPII: rat mast cell protease II

ROS: reactive oxygen species

RP-HPLC: reverse phase high-performance liquid chromatography

RP-OP: peptide fractions of the hydrolysate of ovalbumin with pepsin separated by RP-HPLC

T reg: regulatory T cells

***t*-BOOH:** *tert*-butyl hydroperoxide

TFA: trifluoroacetic acid.

Th: T-helper cell

TLR: toll-like receptor

TSLP: thymic stromal lymphopoietin

1. INTRODUCTION

IMMUNOMODULATING PEPTIDES

IgE-mediated food allergy is a hypersensitivity reaction whose prevalence is rapidly increasing in the western world (Sicherer et al., 2011; Nwaru et al., 2014). Food allergy results from a failure in stablishing oral tolerance or a breakdown in existing tolerance (Burks et al., 2008). Allergic sensitization occurs when antigenic proteins enhance T lymphocyte differentiation into Th2 cells that synthetize cytokines such as IL-4, IL-5 and IL-13. This leads to the activation of B lymphocytes to IgE-producing plasma cells and the binding of the protein-specific IgE antibodies to the surface of tissue mast cells and blood basophils. Fig. 1.1 summarises the main events underlying the immune response to oral allergens in the gut lamina propria and draining mesenteric lymph nodes. Re-exposure to the allergen leads to cross-linking of the cell bound IgE, which triggers the release of mediators responsible for the allergic reaction (Berin and Sampson, 2013). In addition to an individual susceptibility, the ability of proteins or their accompanying food matrix components to promote Th2 effector pathways over Th1 immunity is considered to determine their capacity to induce an allergic response (Berin and Shreffler, 2008). In turn, activation of Th1 cells releases cytokines such as IL-2 and IFN-γ, which elicit the production of pro-inflammatory mediators, TNF-α and IL-8, and reactive oxygen species (ROS), boosting cellular anti-microbial and anti-tumour defense mechanisms (Barnes, 2011). Both types of T cells, with the contribution of Th17, regulatory T (T reg) and regulatory B (B reg) cells, play crucial direct and indirect roles in the maintenance of immune tolerance and suppression of allergic inflammation (Palomares et al., 2010; van de Veen et al., 2013).

Although allergen-specific immunotherapy (IT) has proved effective in allergic asthma and rhinitis, despite huge efforts to desensitize food allergic patients, there are still no validated therapies to induce tolerance or provide effective protection from unintentional exposures, mainly because of the occurrence of severe adverse side effects, the varying efficiency of the traditional allergen extracts used for therapy and the excessive duration of the treatments (Muraro et al., 2014; Nowak-Wegrzyn and Albin, 2015). Thus, in most instances, the

management of food allergy is limited to strict dietary avoidance and emergency treatment in case of adverse reactions.

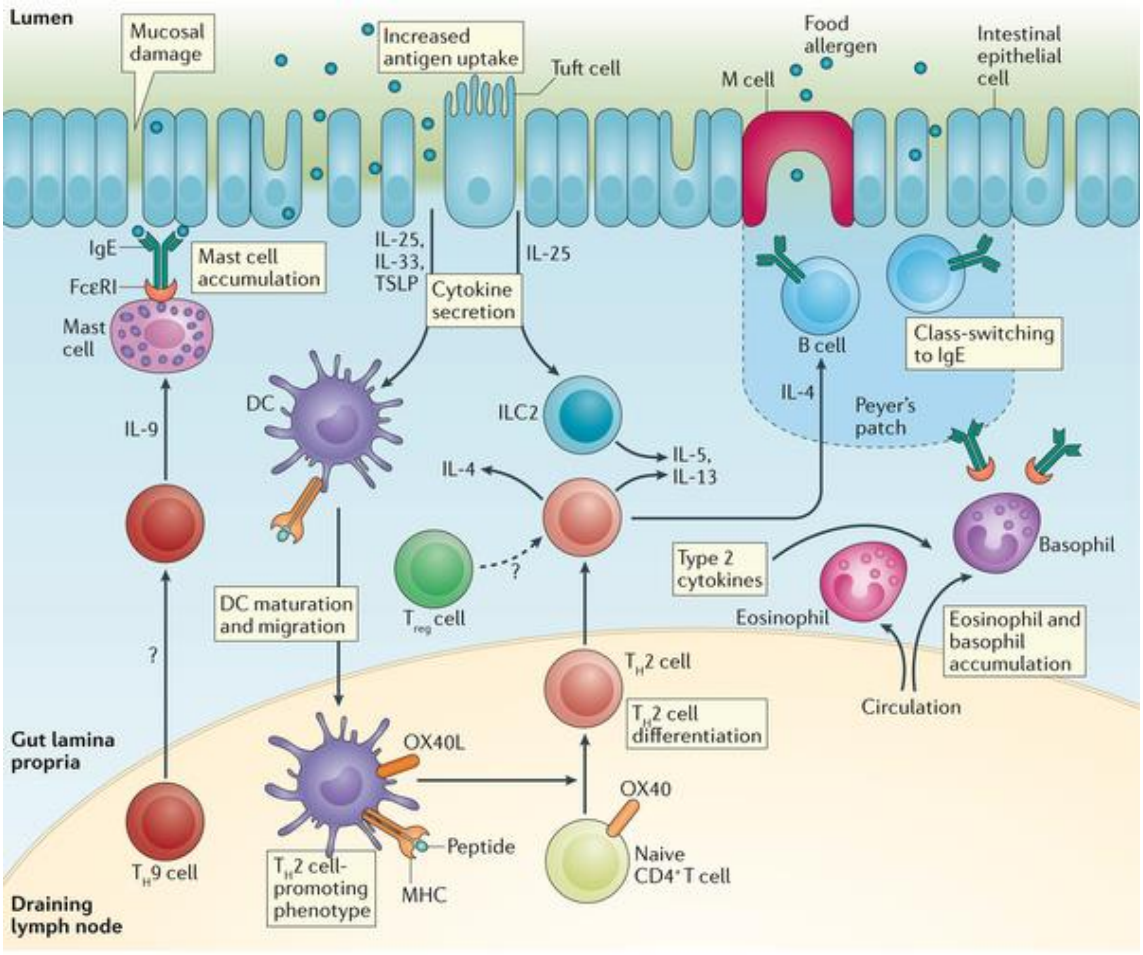

Figure 1.1. Immune response to food allergens. The increased allergen entry promotes the secretion of the epithelium-derived cytokines IL-25, IL-33 and TSLP that trigger the immune system towards a Th2 cell response. In particular, IL-33 and TSLP induces the expression of OX40L in DCs that promotes Th2 cell differentiation of naïve CD4+ T cells and IL-25 secretion aid the expansion of ILC2 population. These events, together with Th2 cytokine secretion (IL-4, IL-5 and IL-13), promote IgE class switching by B cells and the accumulation of eosinophils and basophils. DC, dendritic cell; FcεRI, high-affinity IgE receptor type I; IgE, immunoglobulin E; IL, interleukin; ILC2, type 2 innate lymphoid cells; MHC, major histocompatibility complex; OX40L, (CD252) ligand for CD134; Th, T-helper cell; T reg, regulatory T cell; TSLP, thymic stromal lymphopoietin (reprinted with permission from Nature Publishing Group. Yu et al., 2016).

Among the most promising strategies currently assayed against IgE-mediated allergic diseases stands the possibility of using immunomodulatory peptides to stimulate oral tolerance towards offending food allergens or even to prevent allergic sensitization (Haney and Hancock,

2013; Berin, 2014). Antimicrobial peptides secreted by Paneth cells and peptide hormones secreted by enteroendocrine cells, both crucial for intestinal homeostasis, are good examples of endogenous immunomodulatory peptides. Antimicrobial host defense peptides shape and preserve intestinal microbiota, but are also protective without a direct antimicrobial action because they combine anti-inflammatory and immunostimulatory properties that include effects on cell migration, survival and proliferation, and induction of antimicrobial and immune mediators (Easton et al., 2009). For their part, peptide hormones not only detect luminal nutrients aiding their absorption, but also respond to pathogens and microbial metabolites and exert direct immunomodulatory effects on intestinal immune cells (Worthington, 2015).

Food proteins are a rich and varied source of peptides bearing valuable and diverse biological activities. Bioactive peptides can be released from food proteins *in vivo* by the action of enzymes from digestive fluids and intestinal epithelial cells (IECs) or through processing by antigen-presenting cells. *In vitro*, they can be produced by enzymatic or chemical proteolysis or synthesis. *In vitro* generated peptides need to resist the influence of extreme pH, proteolytic enzymes and surfactants throughout digestion, to reach their sites of action and exert physiological functions. The beneficial effects of immunomodulatory peptides of food origin on food allergy could be directly executed in the intestinal tract, by maintaining and reinforcing barrier function and immune homeostasis, or once being absorbed, through their action on cells of the innate and adaptive immune system. Different IT experiments with food peptides in murine models have been reported, including approaches targeted to achieve desensitization or tolerance and to prevent allergy progression or the development of allergic sensitization.

1.1. Peptides with local effects on the intestinal function

The intestinal epithelium includes 4 main cell types: absorptive enterocytes, goblet cells -responsible for mucus secretion-, enteroendocrine cells and Paneth cells -which secrete, respectively, hormones and antimicrobial peptides-. In addition to its double function to act as a physical barrier with the external environment and to allow the uptake of nutrients, it is now

recognized that the cells that make up this protective interphase drive innate immune responses and ultimately act as modulators of adaptive immunity, therefore playing an essential role in the pathogenesis and development of food allergy. Food proteins and peptides influence the intestinal function by affecting barrier regulation, IEC-signalling to immune cells of the gut associated immune system (GALT) and mucus secretion (Martínez-Augustin et al., 2014). Moreover, the cross-talk of peptides with the microbiota, through prebiotic and antimicrobial actions, although out of the scope of this thesis, is crucial in the regulation of intestinal homeostasis in view of the key role of commensal flora in the development and functioning of the GALT.

The barrier function of the intestine prevents the entrance of pathogens and antigens from the intestinal lumen to the mucosal tissue. In addition to commensal and probiotic bacteria, different components of the diet promote the maintenance of the barrier integrity and thus, contribute to regulate the passage of water and nutrients, and to protect against inflammatory, autoimmune and allergic diseases (Ulluwishewa et al., 2011; Kotler et al., 2013). It has been shown that fish protein hydrolysates can potentiate gut integrity and repair by virtue of both pro-migratory and proliferative activities, as demonstrated on rat (RIE-1) and human (HT29) IECs, and that they are also able to reduce sustained injury in an *in vivo* gastric damaging model (Fitzgerald et al., 2005). Similarly, bovine colostrum hydrolysed with pepsin and chymotrypsin contains protein fractions responsible for promoting the growth of the human intestinal epithelial cell line T84, with potential application in gastrointestinal restoration (Morgan et al., 2014).

On the other hand, milk proteins fed to rodents can strengthen the tight junctions -which seal the space between IECs and control the paracellular transport of water, ions and small molecules- through changes in the expression of transmembrane and/or intracellular proteins that form the junctional complexes. However, while the beneficial effect of whey proteins on intestinal epithelial integrity, attributed to an increased the expression of claudin-4, was mediated by the presence of the anti-inflammatory cytokine TGF-β in the whey protein

preparation (Ozawa et al., 2009; Hering et al., 2011), caseins may increase the expression of the pore-sealing protein claudin-1, and reduce that of the pore-forming claudin-2, through a TGF-β independent pathway (Visser et al. 2010). In fact, casein hydrolysates, unlike amino acid mixtures, were found to reinforce the intestinal barrier function *in vivo* through an effect on the tight junction proteins. This suggests the specific involvement of the peptides contained in the casein hydrolysates, although it remains to be stablished to what extent they modulate intestinal permeability by directly targeting signal transduction pathways, promoting the release of regulatory cytokines, such as IL-10 or TGF-β, stimulating mucin secretion, or inducing interactive support by the gut microbiota (Visser et al., 2012).

For instance, the peptide NPWDQ (α_{S2}-casein 107-111), the active form of GPIVLNPWDQ (α_{S2}-casein 102-111) identified in an enzymatic cheese hydrolysate, upregulates the expression of the occludin gene and the production of the protein (but not of claudin-1) in Caco-2 cells (Yasumatsu and Tanabe, 2010). NPWDQ inhibits the transport of ovalbumin *in vitro*, in Caco-2 cells cultured on filter supports (Tanabe et al., 2006), *ex vivo*, in jejunal and ileal loops, and *in vivo*, following oral feeding of ovalbumin to indomethacin-administered rats (Isobe et al., 2008). A similar activity was reported for DKIHPF (β-casein 47-52), which hinders intestinal permeation of β-lactoglobulin, suggesting a role in the prevention or treatment of food allergy by avoiding allergen passage through the fortification of the intestinal barrier (Tanabe, 2012). Indeed, it has been suggested that augmented intestinal permeability is a factor promoting sensitization to food antigens in susceptible individuals (Heyman, 2005). Furthermore, in sensitized patients, the increase in permeability that is produced as a consequence of occasional food allergic reactions, and contributes to amplify the inflammatory response, does not return to normal values even long after the last allergen exposure (Perrier and Corthésy, 2010).

A contrasting example is that of gliadin, the 33-mer peptide that mediates T cell activation in celiac disease. Gliadin causes rearrangement of the cell cytoskeleton, loss of

occludin-ZO1 protein-protein interaction and increased monolayer permeability in rat (IEC6) and human (Caco-2) IECs (Drago et al., 2006). Enhanced intestinal permeability upon gliadin exposure does not appear to be limited to celiac patients, although an exaggerated increase in gliadin-induced permeability is a characteristic of active celiac disease (Hollon et al., 2015).

The control of epithelial permeability, innate immune recognition and adaptive immunity are all associated with gut inflammation. Perturbations in epithelial or immune homeostasis, either induced by infectious diseases or dietary antigens, can lead to inflammation -an excessive immune activation characterized by an influx of inflammatory cells from the blood- and to increased concentrations of cytokines, free radicals and lipid mediators (MacDonald and Monteleone, 2005). Inflammation represents a critical determinant in the development of food allergy, but also its consequence. In a situation of inflammation, intestinal permeability may be increased and the altered rate, route and mode of antigen presentation may lead to abrogation of oral tolerance (Bischoff et al., 2014). It has recently been demonstrated that the initiation of peanut allergy in mice requires cellular damage or tissue injury that prevent the induction of tolerance through the overproduction of uric acid in the local microenvironment and the subsequent activation of dendritic cells (DCs) (Kong et al., 2015). On the other hand, as already mentioned, inflammation is the long term consequence of sporadic or repetitive exposures to allergens in sensitized patients (Galli et al., 2008).

Food derived peptides can prevent and repair the damage caused by oxidative stress and inflammatory reactions (Sarmadi and Ismail, 2010; Samaranayaka and Li-Chan, 2011; Power et al., 2013). Several publications report antioxidant and anti-inflammatory effects of protein hydrolysates and food peptides on animal models of inflammatory bowel disease (Daddaoua et al., 2005; Lee et al., 2009; López-Posadas et al., 2010; Mochizuki et al., 2010; Espeche Turbay et al., 2012; Wada et al., 2013; Zhang et al., 2015), as well as on different human cell lines (Phelan et al., 2009; Huang et al., 2010; McCarthy et al., 2013 and 2016; Fernández-Tomé et al., 2014; ; Malinowski et al., 2014; Bamdad et al., 2015; Ko et al., 2016). In human IEC models, such as Caco-2 cells, subjected to oxidant stimuli, phosphopeptides from hen egg yolk

phosvitin exhibit antioxidant activity, reducing IL-8 secretion and increasing intracellular glutathione levels and glutathione reductase activity (Katayama et al., 2006). These phosphopeptides also down-regulate the gene expression of IL-8 and IL-12 in lipopolysaccharide (LPS)- and TNF-α-stimulated HT29 cells (Xu et al., 2012). It was suggested that, in addition to the particular peptide structure, the phosphorus content plays a role in their activity and, in fact, casein phosphopeptides also enhance the antioxidant defense systems involved in the glutathione cycle in the Caco-2 cell line (Laparra et al., 2008). Similarly, whey protein hydrolysates inhibit IL-8 secretion and ROS generation, and lunasin (a 43-mer soybean peptide) reduces ROS levels, induced by H_2O_2 and LPS, respectively, in Caco-2 cells (Piccolomini et al., 2012; García-Nebot et al., 2014).

The nuclear factor NF-κB pathway represents a prototypical pro-inflammatory signalling route. The NF-κB group of proteins -which consists of homo- and heterodimeric subunits of the Rel family, including p50 and p65- is found in almost all animal cell types and controls the expression of pro-inflammatory genes involved in the production of cytokines, chemokines, and adhesion molecules. The activity of NF-κB is regulated by IκB kinase (IKK), which retains NF-κB in the cytoplasm complexed with the inhibitory protein IκBα. Upon stimulation, IKK is phosphorylated and, in turn, it phosphorylates the IκBα protein, leading to the dissociation of IκBα from NF-κB and to the activation and translocation of NF-κB to the nucleus, where it binds to its specific promoter elements and induces gene transcription. A variety of integral membrane receptors, such as Toll-like microbial pattern recognition receptors (TLR), switched by microbial products, or receptors which respond to pro-inflammatory cytokines -for instance, TNF-α and IL-1 that are rapidly released on tissue injury or infection- trigger the canonical NF-κB pathway (Lawrence, 2009). Although structurally different, these receptors use similar signal transduction mechanisms, that include phosphorylation of mitogen-activated protein kinases (MAPKs) -like JNK, ERK1/2 and p38, involved in directing cellular responses to a diverse array of stimuli, such as mitogens, ROS and pro-inflammatory cytokines- or activation of IRAK1 -responsible for IL-1-induced upregulation of NF-κB-.

The inhibition of the NF-κB route has long been considered a key goal for anti-inflammatory food components. A 10-mer peptide from durum wheat (QQPQDAVQPF), which reduces cyclooxygenase-2 (COX-2) activity and production of pro-inflammatory cytokines, such as IL-6 and IL-8, triggered by gliadin peptides in the Caco-2 cell model, hampers IRAK1 activation and ERK1/2 and p38 MAPK phosphorylation, and thus, the NF-κB function (Capozzi et al., 2013). The flavour enhancing γ-glutamyl dipeptides, γ-glutamyl cysteine and γ-glutamyl valine, decrease TNF-α-stimulated pro-inflammatory cytokine expression and increase IL-10 expression in Caco-2 cells. It was demonstrated that these peptides inhibit JNK and IκBα phosphorylation through the activation of the calcium-sensing receptor (Zhang et al., 2015). A similar mechanism is followed by the dipeptide WH and by dietary poly-L-lysine, which attenuate the TNF-α-induced secretion of IL-8 from HT29 cells and reduce inflammation in dextran sodium sulphate-induced models of mouse colitis (Kobayashi et al., 2015; Mine and Zhang, 2015). Therefore, the calcium-sensing receptor, present on IECs among other cell types, could constitute a target for treating intestinal inflammation by virtue of its role in maintaining and restoring intestinal homeostasis.

The investigation of immunomodulating food peptides has not only focused on their ability to maintain the integrity of the intestinal epithelial barrier by hindering strong inflammatory responses, but also by inducing the secretion of IgA, which prevents bacterial and toxin translocation and subsequent breakdown of the barrier function (LeBlanc et al., 2002; Otani et al., 2003; Chalamaiah et al., 2014 and 2015). In mice, the oral administration of pasteurized kefir, fish and egg yolk protein hydrolysates increases the number of IgA+ cells, along with the levels of IL-4, IL-6 and IL-10 in the lamina propria -which contribute to B cell activation and antibody production- and raises the IgA content of the intestinal lumen. The secretion of IL-10 is believed to counterbalance the potential actions of the pro-inflammatory cytokines INF-γ and TNF-α, which are also enhanced as a result of these interventions, because peptide administration does not cause tissue damage (Vinderola et al., 2005; Duarte et al., 2006; Nelson et al., 2007; Ndiaye et al., 2012). It is likely that these outcomes are, at least partly,

mediated by the interaction of the peptides with the IECs and the subsequent conditioning of the underlying immune cells (Mallet et al., 2014). Indeed, egg yolk and yellow pea protein derived peptides increase IL-6 secretion by mouse IECs (Nelson et al., 2007; Ndiaye et al., 2012), while casein phosphopeptides induce the expression of IL-6 in Caco-2 and its production in human Int-407 IECs (Kawahara and Otani, 2004; Kitts and Nakamura, 2006).Thus, Nelson et al. (2007) argued that IL-6 produced by IECs as a result of peptide stimulation is enough to trigger B cell differentiation into plasma cells for the production of IgA.

In fact, it is now broadly recognized that the IECs, which constitute the interface between the gastrointestinal contents and the GALT, most likely determine the immune response to food (Berin and Sampson, 2013). IECs release several factors involved in tolerance induction, such as TGF-β, IL-10 and retinoid acid, which drive the differentiation of non-inflammatory DCs and tolerogenic CD103+ DCs that, in turn, induce antigen-specific T reg cells (Coombes et al., 2007; Iliev et al., 2009). Conversely, IECs can act on DCs promoting sensitization through the production of chemokines and cytokines such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 (Chu et al., 2013). In particular, IL-33 regulation in the small intestine was found to be essential for gastrointestinal Th2 priming and subsequent humoral or cellular peanut-induced allergic responses in mice (Chu et al., 2013). On the other hand, TSLP acts directly on T lymphocytes amplifying the activation of Th2 cells and the production of IL-4, IL-5 and IL-13 (Ziegler and Artis, 2010). It has been shown that incubation of Caco-2 cells with the peach allergen Pru p 3 increases the expression of the epithelial cytokines TSLP, IL-33 e IL-25 (Tordesillas et al., 2013). Similarly, thermal processing of the major peanut allergen Ara h 2 results in structural changes which impact its interactions with IECs and the subsequent cellular signalling, enhancing its immunogenicity (Starkl et al., 2011).

It is speculated that, as opposed to allergenic proteins, certain factors derived from the diet and the microbiota, with direct access to the IECs, could indirectly prime DCs in the control of the immune response, stimulating tolerance rather than activating the development of an allergic phenotype (Berin and Shreffler, 2008). Leonard et al. (2012) described that, in mice, the

protection against anaphylaxis that arises from oral IT (OIT) is associated with significant changes in the intestinal expression of certain genes. This observation denotes the existence of a protective mechanism located in the intestinal mucosa, whose activation would depend on interaction and communication with IECs, and suggests that it may be switched by the stimulus of immunomodulating peptides. However, it remains to be elucidated to what extent food derived peptides exhibit intrinsic immunogenicity by interacting with IECs.

The mucus layer that covers the intestinal epithelium, in addition to offering physical protection against mechanical, chemical and microbial challenges, provides it with enzymatic activities and filter properties that affect the availability of antigens for entering the mucosa. In this respect, several food protein hydrolysates and peptides have shown stimulatory effects on mucin secretion in rat jejunum that are linked to a structure favourable to binding to opioid receptors (Claustre et al., 2002; Trompette et al., 2003). Further research demonstrated the induction of mucin production by peptides derived from caseins and whey proteins (such as YPFPGPI -β-casein 60-66-, YGLF-NH_2 -β-lactoglobulin 102-105-, YLLF-NH2 -α-lactalbumin 50-53- and AYFYPEL -α_{S2}-casein 143-149-) through the modulation of the expression of mucin genes in human HT29-MTX goblet IECs (Zoghbi et al., 2006; Martínez-Maqueda et al., 2012 and 2013). In particular, peptide sequences with the ability to stimulate the production of Mucin 2 (Muc2), such as β-casein 94-123 (Plaisancié et al., 2015), could play a tolerogenic role. Muc2 -the major component of the mucus layer of the intestine- exerts anti-inflammatory properties on IECs and DCs (Shan et al., 2013). Furthermore, it has been shown that goblet cells of the small intestine can take up luminal material and deliver it to the tolerogenic CD103+ lamina propria DCs, with recent observations pointing at a coupling between goblet cell antigen uptake and mucus secretion (McDole et al. 2012; Pelaseyed et al., 2014). Consequently, Muc2 enhances gut homeostasis and tolerance by suppressing inflammatory responses in DCs while imprinting them with a tolerogenic function (Shan et al., 2013).

1.2. Intestinal transport of peptides

The passage of immunomodulating peptides across the intestinal barrier is a requirement for direct conditioning of the underlying immune cells of the GALT, as well as for systemic effects. As compared with intact proteins, hydrolysis products are endowed with distinct transport properties, both in terms of rate and pathway (Bernasconi et al., 2006). Fig. 1.2 shows possible routes of antigen delivery across the epithelial barrier. Food peptides can use the antigen sample pathway, that involves microfold cells (M cells) located above isolated lymphoid follicles and Peyer's patches (PP), which allows the trafficking of insoluble or particulate substances, although other transport mechanisms -such as the already mentioned paracellular passage regulated by tight junctions, passive diffusion through IECs, endocytosis and carrier-mediated transport systems- are also available to deliver soluble antigenic molecules to the lamina propria. Whereas there seems to be little unequivocal evidence that dietary bioactive peptides, other than di- and tri-peptides, are absorbed at physiologically relevant concentrations to produce systemic actions (Miner-Williams et al., 2014), the possibility exists that immunomodulating peptides can exert certain effects, at least those mediated by their interaction with cells of the adaptive immune system, at small concentrations, within the range of those required by large dietary antigens to induce tolerance.

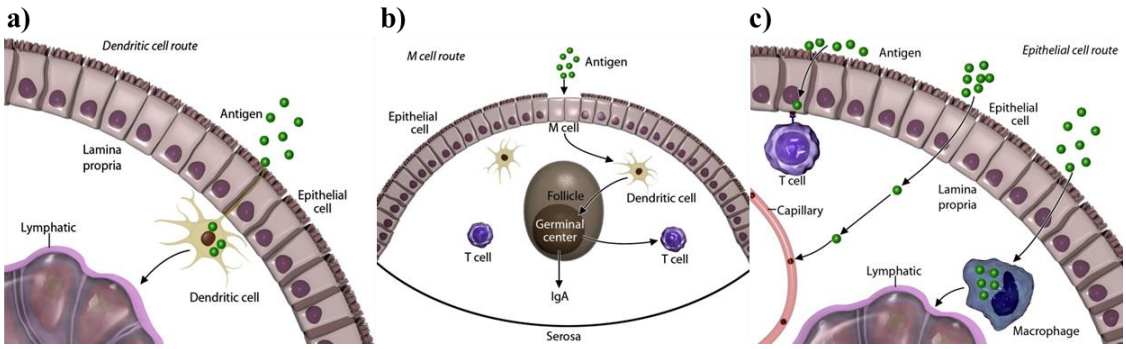

Figure 1.2. Passage of antigens across the epithelial barrier in the gut. (a) DCs extend processes through the epithelium and into the lumen. (b) M cells overlying Peyer's patches take up particulate antigens and deliver them to subepithelial DCs. (c) Soluble antigens also cross the epithelium through transcellular or paracellular routes to encounter DCs and T cells in the lamina propria. DCs, dendritic cells; IgA, immunoglobulin A (Modified with permission from Elsevier. Burks et al., 2008).

Interestingly, it was found that IECs may constitute a powerful link between luminal antigens and local immune cells by mediating the transfer of only small amounts of soluble proteins or peptides. Most (90%) of the protein material that is taken by IECs is either totally (50%) or partially (40%) degraded in endosomes, yielding, in the latter case, peptides of around 1500 Da of molecular mass, a size compatible with binding to major histocompatibility complex class II (MHC-II) molecules. In IECs, as in professional antigen-presenting cells, endosomes containing partially degraded antigens may encounter MHC-II compartments, what allows peptides to be loaded on MHC-II molecules. This gives rise to exosome-like vesicles that very efficiently transfer MHC-II-peptide complexes to the lamina propria, where they can interact with local DCs and stimulate specific T cells at a concentration 100 times lower than free peptides (Ménard et al., 2010). Thus, the human IEC lines HT29 and T84 secrete exosomes bearing accessory molecules which strongly potentiate peptide presentation to T cells (Van Niel et al., 2001; Mallegol et al., 2007). However, it has been reported that, in mice, epithelial exosomes prime for immunogenic rather than tolerogenic responses (Van Niel et al., 2003), although the outcome would definitely depend on the phenotype of the local DCs and on the signals delivered by the IECs themselves and by exogenous factors, such as inflammatory stimuli. Indeed, exosomes derived from the serum of ovalbumin fed mice were found to prevent allergic sensitization in a model of allergic asthma, with the tolerant animals showing a significantly higher frequency of activated T reg cells (Almqvist et al., 2008).

On the other hand, in sensitized individuals, CD23 (FcεRII) -also called the low-affinity receptor for IgE- expressed by IECs and B cells, moves IgE and antigen-IgE complexes across the epithelium by transcytosis, exerting both negative and positive effects in the regulation of IgE production (Tu et al., 2005). This carrier-mediated transport enables, in cases of peptides with adequate length, activation of the high-affinity receptor of IgE, FcεRI, on mast cells and promotion of allergic inflammation, secretion of IL-4 and IL-13, local IgE production, facilitated antigen presentation by B cells and sensitization to additional antigens (Galli and Tsai, 2012). However, and as it will be explained in the following sections, since FcεRI is also

expressed by human DCs, its activation by peptide-IgE complexes can influence, maturation, functional activation, migration of DCs and antigen presentation with a beneficial impact in tolerance induction (Platzer et al., 2015).

1.3. Effects on cells of the innate immune system: anti-inflammatory peptides

It has been reported that the oral administration of several food protein hydrolysates can activate the effector mechanisms of the innate immune response. For example, the phagocytic activity of peritoneal macrophages, and consequently their ability to respond to pathogens, increases after the administration to mice of hydrolysates of milk, fish and yellow field pea proteins (Biziulevicius et al., 2006; Duarte et al., 2006; Ndiaye et al., 2012; Chalamaiah et al., 2014).

Macrophage activation leads to an inflammatory response. Inflammation is part of the defence mechanism against infectious agents and injuries, but it is also associated to adverse chronic conditions such as cardiovascular diseases, diabetes and cancer. Macrophages are activated in response to oxidative stress or pro-inflammatory cytokines, including IFN-γ, and by bacterial components, such as the TLR ligand LPS, resulting in the production of the pro-inflammatory substances: IL-1β, IL-6, TNF-α, nitric oxide (NO) and prostaglandin E2 (PGE2). NO and PGE2 are generated during inflammatory processes by inducible nitric oxide synthase (iNOS) and COX-2 -which catalyse, respectively, the reactions that convert L-arginine and oxygen to NO and citruline, and arachidonic acid to prostaglandins-. In this respect, and as shown in Table 1.1, a number of food-protein hydrolysates and derived peptides, particularly from soy, fish and shellfish, have shown anti-inflammatory properties on LPS-stimulated murine RAW 264.7 cells, as estimated by different markers associated with beneficial effects against inflammation and oxidative stress. The RAW 264.7 cell line is considered one of the most suitable *in vitro* model systems for inflammation-related investigations. In addition, other food protein hydrolysates have shown anti-inflammatory properties in human cell lines, such as

monocyte (THP-1) derived macrophages (Millán-Linares et al., 2014; Montoya-Rodríguez et al., 2014).

The cascade of LPS-induced events in macrophages is initiated by its binding to the LPS-binding protein and transfer to CD14 on the macrophage surface for further interaction with TLRs. Through MAPKs, TLRs activate the already mentioned NF-κB pathway. A mechanism of action involving the inhibition of NF-κB activation, leading to the down-regulation of genes implicated in inflammatory processes in RAW 264.7 cells, such as COX-2, iNOS, IL-6, IL-1β or TNF-α, has been described for lunasin and lunasin derived peptides (de Mejía and Dia, 2009). A reduced transcriptional activity of NF-κB has also been deemed responsible for the antioxidant activity of hydrolysed common bean and soybean flours and amaranth hydrolysates (Oseguera-Toledo et al., 2011; Vernaza et al., 2012; Montoya-Rodríguez et al., 2014). Similarly, the anti-inflammatory properties of pyroglutamyl-leucine in the RAW 264.7 cell line are probably due to the inhibition of NF-κB, via suppression of the phosphorylation of three types of MAPKs: JNK, ERK and p38, and of its dissociation from IκBα (Hirai et al., 2014).

In addition to interfering with TLR signal transduction pathways, there are also peptides which supress LPS-induced activation of macrophages through interaction with LPS itself and neutralization of its action. Antimicrobial host defence peptides, which are generally small (commonly 12-50 amino acids) cationic and, frequently, quite hydrophobic and amphipathic, interact with the negatively charged surface of LPS and suppress the LPS-triggered release of pro-inflammatory cytokines from macrophages (López-Abarrategui et al., 2013).This is, for instance, the case of antimicrobial peptides derived from shrimp, such as the shrimp anti-LPS cyclic peptide (ECKFTVKPYLKRFQVYYKGRMWCP) (Lin et al., 2010).

Table 1.1. Food protein hydrolysates and derived peptides with anti-inflammatory activity in the macrophage RAW 264.7 cell line

Peptide/hydrolysate	RAW 264.7 stimulation	Main outcomes	Reference
Lunasin	Lunasin pre-treatment for 6 h and LPS stimulation for 18 h	↓ROS, ↓TNF-α (23%) and ↓IL-6 (37%) production	Hernández-Ledesma et al., 2009
Lunasin-like peptide of 5000 Da	Simultaneous lunasin treatment and LPS stimulation for 24 h	↓NO (IC_{50}= 28 μM), ↓PGE2 (IC_{50}= 41 μM), ↓ IL-6 (IC_{50}= 2 μM) and ↓ IL-1β (IC_{50}= 13 μM) production COX-1 (no change), ↓iNOS (IC_{50}= 37 μM) and ↓COX-2 (IC_{50}= 25 μM) protein expression ↓NF-κB (IC_{50}= 21 μM) transactivation ↓P65 (IC_{50}= 48 μM) and ↓P50 (IC_{50}= 77 μM) nuclear translocation	de Mejía and Dia, 2009
Soy flour hydrolysed with alcalase	Simultaneous hydrolysate treatment and LPS stimulation for 24 h	↓NO (18-35%) and ↓PGE2 (47-71%) production ↓iNOS (31-53%) and ↓COX-2 (30-52%) protein expression	Martínez-Villaluenga et al., 2009
Common bean flours hydrolysed with alcalase	Simultaneous hydrolysate treatment and LPS stimulation for 24 h	↓NO (IC_{50}= 4-48 μM) and ↓PGE2 (IC_{50}= 14-61 μM) production ↓iNOS (IC_{50}= 5-14 μM) and ↓COX-2 (IC_{50}= 35-44 μM) protein expression ↓NF-κB transactivation ↓P65 nuclear translocation	Oseguera-Toledo et al., 2011
Germinated soybean flour hydrolysed with alcalase	Hydrolysate pre-treatment for 24 h and LPS stimulation	↓NO (21-69%), ↓PGE2 (64-88%) and ↓TNF-α (94-96%) production ↓iNOS (23-94%) and ↓COX-2 (36-77%) protein expression	Vernaza et al., 2012
Commercially available soy products hydrolysed with pepsin and pancreatin	Simultaneous hydrolysate treatment and LPS stimulation for 24 h	↓NO (16-29%), ↓IL-1β (22-40%), ↓IL-6 (27-43%) and ↓TNF-α (48-333%) production ↓iNOS (65-88%) and ↓COX-2 (41-70%) protein expression	Dia et al., 2014
Yellow pea proteins hydrolysed with thermolysin of less than 3000 Da	Hydrolysate pre-treatment for 12 h and LPS-IFN-γ stimulation for 24 h	↓NO (20%), ↓TNF-α (35%) and ↓IL-6 (80%) production	Ndiaye et al., 2012
Phosphopeptides derived from partial alkaline dephosphorylation and tryptic digestion of egg yolk phosvitin	Peptide pre-treatment for 2 h followed by LPS stimulation for 6 h	↓IL-6, ↓IL-1β, ↓TNF-α and ↓iNOS gene expression	Xu et al., 2012
GCQQAVQSAV derived from clam (*Ruditapes philippinarum*) hydrolysed with alcalase	Peptide pre-treatment for 4 h followed by LPS stimulation for 18 h	IL6 (no change), PGE2 (no change) and ↓NO (48%) production ↓iNOS and ↓COX-2 protein expression	Lee et al., 2012
QCQCAVEGGL derived from Pacific oyster (*Crassostrea gigas*) hydrolysed with Protamex	Simultaneous peptide treatment and LPS stimulation for 18 h	↓NO (72%) production	Hwang et al., 2012

GVSLLQQFFL derived from mussel (*Mytilus coruscus*) hydrolysed with Flavourzyme	Simultaneous peptide treatment and LPS stimulation for 18 h	↓NO (62%) production	Kim et al., 2013
Almond proteins hydrolysed with pepsin and pancreatin and the fraction higher than 5000 Da	Simultaneous peptide treatment and LPS stimulation	↓NO production ↓TNF-α, ↓IL-1β, ↓ IL6 production and gene expression ↓iNOS and ↓COX-2 protein and gene expression	Udenigwe et al., 2013
Gluten-derived pyroglutamyl-leucine	Simultaneous peptide treatment and LPS stimulation for various periods	↓NO, ↓TNF-α and ↓IL6 production ↓IκBα degradation ↓JNK, ↓ERK and ↓p38 phosphorylation	Hirai et al., 2014
Extruded amaranth hydrolysed with pepsin and pancreatin	Simultaneous peptide treatment and LPS stimulation for 24 h	↓PGE2 (32%) and ↓TNF-α (34%) production ↓COX-2 (67%) protein expression ↓IKK (88%) and ↓IκBα (66%) phosphorylation	Montoya-Rodriguez et al., 2014
Tuna hydrolysed with alcalase and synthetic peptides PRRTRMMDGGR and MGPAMMRTMPG	Hydrolysate or peptide pre-treatment for 24 h and LPS stimulation for 24/28 h	↓IL-2, ↓TNF-α and ↓TNF-γ production	Cheng et al., 2015
Salmon myofibrillar protein conjugated with alginate hydrolysed with pepsin and trypsin	Simultaneous hydrolysate treatment and LPS-IFN-γ stimulation for 24 h	↓NO (66%), ↓IL-6 (85%) and ↓TNF-α (67%) production COX-1 (no change), ↓iNOS, ↓IL-6, ↓COX-2 and ↓TNF-α gene expression	Saigusa et al., 2015
Fermented (*Lactobacillus plantarum*) abalone (*Haliotis discus hannai Ino*)	Hydrolysate pre-treatment for 24 h and H_2O_2 stimulation Simultaneous or hydrolysate pre-treatment (18 h) and LPS stimulation for 24/28 h	↓ROS (IC_{50}= 6.78 mg/mL), ↓NO and ↓PGE2 production	Hasnat et al., 2015
KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAG DSDGDGKIGVDEFAALVK and AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGD SDGDGKIGVDEFAALVK derived from tilapia (*Oreochromis niloticus*) hydrolysed with Flavourzyme	Peptide pre-treatment for 1 h and H_2O_2 stimulation Simultaneous peptide treatment and LPS stimulation for 48 h	↓ROS (83%) and ↓NO (40.9%) production	Kangsanant et al., 2015
PAY derived from salmon hydrolysed with pepsin	Simultaneous peptide treatment and LPS stimulation for 24 h	↓NO (64%), ↓PGE2 (45%), ↓IL-6 (44%), ↓TNF-α (58%) and ↓IL-1β (65%) production ↓iNOS (0.75-fold) and ↓COX-2 (0.48-fold) protein expression	Ahn et al., 2015

Main outcomes: COX-1=cyclooxygenase-1; COX-2=cyclooxygenase-2; IκBα= nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK= IκB kinase; iNOS= inducible nitric oxide synthase; JNK, ERK and p38= mitogen-activated protein kinases; NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2= prostaglandin E2; p50/p65= members of the Rel family of transcription factors; ROS= reactive oxygen species; ↑= increased or high levels; ↓= decreased or low levels

In contrast to the previously mentioned examples, wheat gliadin and its proteolytic fragments arising from pepsin digestion, such as VSFQQPQQQYPSSQ and FQQPQQQYPSSQ, have the ability to activate mouse peritoneal macrophages inducing the secretion of TNF-α and NO (Tuckova et al. 2002). Gliadin and its fragments also increase IL-8 and TNF-α production by human monocytes, what was suggested to be involved in the damage they cause to celiac intestinal mucosa. Unlike soy proteins, gliadin stimulates phosphorylation and subsequent degradation of the IκBα molecule and increases the DNA-binding activities of the NF-κB subunits p50 and p65, with an activation pathway independent of CD14 (Jelinkova et al., 2004).

It should be mentioned that it has been hypothesized that the suppression of Th1 responses by antioxidant compounds, while beneficial in reducing the side effects of inflammatory processes, may provoke an up-regulation of Th2 type cytokines that promotes allergic sensitization and exacerbates allergic symptoms (Gostner et al., 2014). However, when the role of oxidative stress in allergic sensitization was investigated in humans, it was shown that it is the exposure to allergenic proteins combined with an inadequate antioxidant response what increases the likelihood for sensitization (Utsch et al., 2015). In fact, there is evidence that antioxidant and anti-inflammatory compounds that decrease the effects mediated by IFN-γ and slow down the intracellular pathways that lead to the generation of ROS, also reduce the expression of Th2 cytokines and IgE levels in response to food allergens in mice (Singh et al. 2011; Masilamani et al. 2012). For instance, dietary omega-3 fatty acids prevent allergic sensitization in mice via suppression of Th2 type antibody responses and enhancement of intestinal and systemic Foxp3+ T reg cells (van den Elsen et al., 2013a). Noteworthy, the suppression of IL-4 and IL-13 secretion by mast cells, that contributes to decrease the susceptibility to develop allergy and the severity of the symptoms, is associated with the inhibition of the generation of ROS (van den Elsen et al., 2013b).

In line with those observations, it was reported that the fraction smaller than 3000 Da from an enzymatic hydrolysate of the edible microalgae *Spirulina maxima* and, particularly, the

pentapeptides DAVNR and MMLDF, inhibit histamine release form RBL-2H3 mast cells sensitized with dinitrophenyl (DNP)-specific IgE and stimulated with DNP-BSA, in parallel with a suppressive effect on intracellular ROS production (Vo et al., 2013). Furthermore, the peptide PFNKGTFAS, derived from a gastrointestinal digest of abalone (*Haliotis discus hannai*), attenuates histamine release and production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from human HMC-1 cells. PFNKGTFAS hinders the phosphorylation of JNK and IκBα in the stimulated mast cells, which points at the inhibition of the MAPK and NF-κB pathways as responsible for the restricted release of mediators (Ko et al., 2016). These results indicate that the ability of food peptides to favourably balance pro-inflammatory and anti-inflammatory/antioxidant responses could provide a tolerogenic milieu. In addition, it has been described that there are peptides able to interact with the ε-heavy chain of IgE and block its binding to the high-affinity receptor, FcεRI, on mast cells and basophils and protect mice from anaphylaxis (Zhou et al., 2013). These actions of peptides on mast cells and IgE signalling are highly interesting in view of the role of armed mast cells as effectors of anaphylaxis, but also as inducers of Th2 responses and suppressors of T reg cells (Burton et al., 2014).

1.4. Effects of peptides on cells of the adaptive immune system: Th1 and Th2 mediators

As already anticipated, there is evidence that immunomodulating peptides alter T lymphocyte functions and can contribute to a balanced Th1 and Th2 response, with potential implications in various clinical conditions, including allergy and oral tolerance to antigens (Kiewiet et al., 2015).

Antigen-presenting cells, such as DCs, are key initiators of innate immunity, although they also play a central role in shaping the properties of adaptive immunity by processing proteins or peptides and presenting them to T cells, together with co-stimulatory mediators that generate primary T cell responses (see Fig. 1.3, which outlines the main cytokines and transcription factors involved in T cell differentiation). As shown in Fig. 1.2., food antigens can

be directly sampled from the gut lumen by intestinal DCs, which extend their dendrites between IECs. Alternatively, they can be transported through M cells into the lymphoid follicles, or by transcytosis to the lamina propria, and presented by DCs to naïve T cells in the PP or the gut-draining mesenteric lymph nodes (MLN) (Ménard et al., 2010). As already indicated, intestinal DCs, with the help of other cells involved in innate immunity, such as the nearby IECs, play a crucial role in the generation of tolerance to food proteins (Ruiter and Shreffler, 2012). Thus, while allergens, as well as certain food components, may exhibit intrinsic adjuvant capacity, stimulating DCs to enhance allergen-specific Th2 differentiation (Kean et al., 2006; Shreffler et al., 2006; Hsu et al., 2010; Gómez et al., 2012; Chu et al., 2013), it is also possible to prime DCs for allergy prevention or treatment. For instance, soybean isoflavones hinder allergic sensitization by inhibiting DC maturation and subsequent DC-mediated Th2 effector cell functions, which correlates *in vivo* with significantly reduced anaphylactic symptoms and mast cell degranulation upon allergen challenge (Masilamani et al., 2011). Accordingly, DCs are attractive targets for tolerance induction and the potential of food peptides to avoid Th2 type immunity by the enhancement of the tolerogenic properties of DCs warrants further investigation.

It is known for some time that FcεRI, in addition to mast cells and basophils, is also constitutively expressed on DCs and monocytes in humans, albeit not in mice (Shin and Greer, 2015). Experiments with transgenic mice led to postulate that FcεRI contributes to the rapid internalization of IgE-bound antigens by DCs, facilitating their presentation to specific T cells *in vivo* and promoting the development and activation of Th2 cells (Sallmann et al., 2011). However, other studies showed that, when monovalent antigens, unable to cross-link IgE-FcεRI complexes, are loaded by DCs, enhanced presentation results in a transient T cell proliferation, followed by systemic deletion of antigen-specific T cells (Baravalle et al., 2014). This mechanism, if confirmed in further studies, implies a role for peptides derived from allergenic proteins, focused by IgE-binding and transported by CD23, in the development of tolerance through the mediation of DCs. Furthermore, it was recently found that even antigen-specific

IgE-FcεRI cross-linking fails to induce maturation and production of inflammatory mediators by human and FcεRI-humanized DCs *in vitro*, which correlates with a reduced severity of food allergy *in vivo* (Platzer et al., 2015). These observations indicate that DC-intrinsic IgE signals function as feedback mechanisms to control allergic inflammation and suggest novel beneficial peptide-based approaches for allergy therapy.

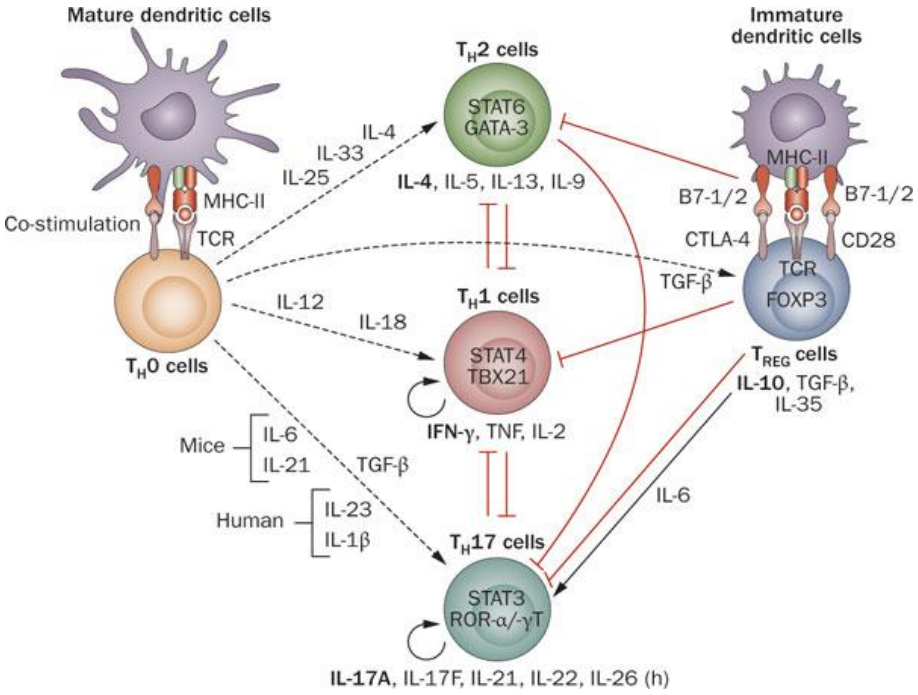

Figure 1.3. T cell differentiation: cytokines and transcription factors. Mature DCs polarize naive Th0 cells into different T cell subsets (Th1, Th2, Th17 or T reg) through several signals, including antigen presentation to the TCR, secretion of cytokines and co-stimulatory signals. The archetypical cytokine of each subset is marked in bold. The complex interactions between the various CD4+ T cell subsets are represented schematically. Immature DCs also prime T reg cells to induce regulatory signals. CTLA-4, cytotoxic T lymphocyte antigen 4; DCs, dendritic cells; Foxp3, forkhead box protein P3; GATA-3, trans-acting T-cell-specific transcription factor; IL, interleukin; MHC, major histocompatibility complex; ROR, retinoic acid-related orphan receptor transcription factor; STAT, signal transducer and activator of transcription; TBX21, T-box transcription factor (also known as T-bet); TCR, T-cell receptor; Th, T-helper cells; T reg, regulatory T cells (reprinted with permission from Nature Publishing Group. Lahoute et al., 2011).

As opposed to IgE-mediated food allergies, in the pathogenesis of celiac disease, and in addition to the inflammatory innate immune response that causes damage to the intestinal mucosa, the adaptive immune response to gliadin involves DCs that interact with gliadin-specific Th1 cells, producing inflammatory cytokines such as IFN-γ (Green et al., 2015).

Stimulation of DCs with gliadin, unlike other food proteins, enhances the expression of maturation markers (CD80, CD83, CD86, and HLA-DR molecules) and the secretion of chemokines and cytokines (IL-6, IL-8, IL-10, TNF-α, growth-related oncogene, etc.), which results in more efficient processing and presentation of gliadin peptides to specific T cells (Palova-Jelinkova et al., 2005). However, two decapeptides naturally occurring in durum wheat, QQPQDAVQPF and its homologous QQPQRPQQPF, are able to prevent the gliadin-dependent functional DC maturation and T cell stimulating capacity, raising the perspective of a potential therapeutic strategy alternative to a gluten free diet (Giordani et al., 2014).

Peptides from marine origin administered orally to mice enhance the proliferative response of spleen cells to concanavalin A (ConA) and the percentage of CD4+ T cells (Yang et al., 2009a) or both CD4+ and CD8+ T cells (Chalamaiah et al., 2014). In addition, several studies describe that food peptides (Kawahara et al., 2004; Jiehui et al., 2014) and hydrolysates (Mercier et al., 2004; Durrieu et al., 2006; Mao et al., 2007; Saint-Sauveur et al., 2008; Kong et al., 2008; Yang et al., 2009a; Hou et al., 2012; He et al., 2015) promote the proliferation of either resting or mitogen-treated murine spleen cells and human T cells *in vitro*, which was also interpreted as a stimulatory effect on cell-mediated immunity. However, in most cases, those works assessed cell viability -the number of healthy cells in a sample- rather than cell proliferation -the number of dividing cells-.

The results available so far suggest that, beyond influencing proliferation, immunomodulating peptides can specifically drive lymphocyte responses in a certain direction. Milk protein hydrolysates have been reported to induce the secretion of Th1 cytokines (IL-2 and IFN-γ) by ConA-stimulated mice spleen cells and human Jurkat T cells, without an effect on the release of Th2 cytokines and thus, to play a role in fighting infections (Mao et al., 2007; Saint-Sauveur et al., 2008; Phelan et al., 2009). Conversely, the casein peptide QEPVL enhances Th2 type responses *in vivo*, as estimated by the concomitant up-regulation of IL-4 and down-regulation of IFN-γ and TNF-α in mice serum, and inhibits LPS-induced inflammation (Jiehui et al., 2014). These observations imply that food peptides may preferentially guide Th1 type

immune responses or promote Th2 type responses, which are goals of recognized importance in infectious diseases, tumours and inflammatory processes.

With regard to food allergy, it should be noted that the therapeutic effect of peptides in mouse models has been associated both to the inhibition of allergen-induced Th1 and Th2 cytokine responses (Yang et al., 2009b), as well as to the promotion of Th1-biased responses to a detriment of the Th2 ones (Yang et al., 2010). Alternatively, other immunomodulating peptides could help to induce oral tolerance by stimulating T reg cells through the promotion of the secretion of regulatory cytokines, such as IL-10 (Prioult et al., 2004; Cian et al., 2012) or TGF-β (Rodriguez-Carrio et al., 2014).

1.5. Peptide immunotherapy: T and B cell epitopes

The production of allergen derivatives that are hypoallergenic and immunogenic is an attractive strategy for the development of IT agents for IgE-mediated allergies. Although its efficacy has not been formally demonstrated, OIT -which consists in the gradual administration of increasing amounts of the allergen- is one of the most promising treatment options. However, the associated high frequency of allergic adverse events has prompted the investigation of strategies aimed at reducing the allergenicity of the treatment preparations, while maintaining their immunogenicity, or even improving their immunomodulating potential, to achieve effective desensitization or sustained oral tolerance (Jutel et al., 2015; Vázquez-Ortiz and Turner, 2016; Wood, 2016).

Peptide IT focuses on the properties of fragmented allergens, which contain T cell-stimulating epitopes but are not capable of cross-linking IgE on basophils and mast cells. Peptides represent a safer alternative to full allergens, as they produce fewer side effects and increase adherence to therapy (Casale and Stokes, 2011; Moldaver and Larché, 2011). T cell epitope-mapping -that is the determination of the specific peptide sequences recognized by CD4+ T cells- can be performed by different methods but, in all cases, it needs to be checked that as many as possible distinct MHC-II molecules, representative of the HLA genotypic

frequencies of the patient population of interest, recognize and bind the selected epitopes (Malherbe, 2009).

T cell epitopes that can be potential candidates for peptide-based vaccines have been identified from many food allergens, but particularly from peanut, *in silico* -by the use of predictive algorithms- and *in vitro* -by screening the proliferative responses and cytokines released by peripheral blood mononuclear cells (PBMCs) from allergic patients stimulated with consecutive overlapping peptides spanning the allergen sequence or by tetramer-guided epitope mapping- (DeLong et al., 2011; Prickett et al., 2011 and 2013; Pascal et al., 2013). Allergen-specific T cell lines or clones have been used to overcome the limitation imposed by the low frequency or allergen-specific T cells (DeLong et al., 2011; Prickett et al., 2011 and 2013), although *in vitro* expansion can alter cell phenotypes or bias the results through the selection of the rapidly proliferating clones (Pascal et al., 2013). Specific T cell epitopes of food allergens, such as celery (Bohle et al., 2003), hazelnut (Bohle et al., 2005), shrimp-tropomyosin (Wang et al., 2012; Ravkov et al., 2013), β-lactoglobulin (Inoue et al., 2001; Kondo et al., 2008), α_{S1}-casein (Ruiter et al., 2006), ovomucoid (Holen et al., 2001), peach Pru P3 (Tordesillas et al., 2009; Pastorello et al., 2010), anisakis (Garcia Alonso et al., 2015) and walnut (Archila et al., 2015), among others, have also been described.

There is a minimum core sequence of 9 to 15 amino acids required for recognition by reactive T cells. Consequently, -even if linear, soluble, 15-mer IgE-binding peptides are unlikely to act as full epitopes and cause allergic reactions (Albrecht et al., 2009)- the selection of peptides that combine the ability to stimulate T lymphocytes and modulate the immune response with a reduced capacity to trigger clinical symptoms implies a compromise, as longer peptides increase T cell epitope coverage but also the potential for IgE-cross-linking (Prickett et al., 2011 and 2013). The lack of overlapping between T- and IgE-binding epitopes has to be evaluated by searching B epitopes reported in the literature, and confirmed by serum IgE-binding and basophil activation assays (Wai et al., 2014). Peptide microarray technology allows the assessment of epitope diversity and affinity of the IgE response associated to clinical reactivity

(Berin, 2015). Unfortunately, the evidence gathered so far highlights that B epitope multiplicity and variation in reactivity among allergic individuals is so large that the presence or absence of specific epitopes might not provide enough evidence for allergy prediction (Aalberse and Crameri, 2011; Ruiter and Shreffler, 2012; Scheurer et al., 2015).

Cytokine detection assays typically reveal a prevalent Th2 cytokine profile in PBMCs or specific T cells from allergic patients stimulated with the T cell epitopes regarded as most significant, while control donors show a trend towards a Th1 profile (Tordesillas et al., 2009; Pastorello et al., 2010; DeLong et al., 2011; Prickett et al., 2011; Pascal et al., 2013). Nonetheless, beyond trying to reduce IgE reactivity and retain T cell reactivity, the possibility arises to exploit the immunomodulatory potential of peptides, in particular the ability to re-direct the immune response of specific cell types and the lack of inflammatory properties, to enhance the effectiveness and reduce the length of the IT treatments. In this respect, B cell epitope therapy, which uses non IgE-reactive peptides derived from the IgE-binding sites of allergens -with a diminished potential to release Th2 cytokines and inflammatory mediators, but able to induce allergen-specific blocking IgG antibodies when fused to the appropriate carrier protein- can make IT safer and more efficient (Valenta et al., 2016). Regarding food allergies, as it will be explained in the next section, mouse experiments have shown that peptides that do not induce Th2 cytokines and/or are able to increase allergen-specific IgG can play a valuable therapeutic role by providing and extra-help to deviate predominantly Th2 to Th1 responses, by inducing T reg cells that downregulate both Th2 and Th1 responses, or by blocking allergen binding to IgE (Prioult et al., 2005; Yang and Mine, 2009; Wai et al., 2014 and 2016).

Generally, many distinct fragments of food allergens are able to generate T and B cell responses (Prickett et al., 2011; Berin, 2015; Wai et al., 2016), although the final effect might depend on the occurrence of the right combination of peptides with synergistic or adjuvant actions, or on the peptides being in an aggregated state resembling the intact molecule (Yang et al., 2010; Bøgh et al., 2012; Meulenbroek et al., 2013). On this basis, enzymatically hydrolysed allergens are a practical alternative to synthetic dominant T cell epitopes specific for each HLA

type -as the former would contain enough peptides to address MHC-II diversity in patients- that could also provide non IgE-cross-linking B epitopes (Knipping et al., 2012; Kulis et al., 2012). Hydrolysis of food proteins emerges as an attractive and safe means for a reproducible and standardized production of immune-stimulating peptides at low cost, as compared with that associated with generating synthetic peptides on a large scale.

Immunodominant T cell epitopes with negligible IgE-binding and lacking inflammation stimulating capacity have been used to modify beneficially the immune reaction to allergens in clinical trials of IT by subcutaneous injection, demonstrating persistent efficacy with few adverse events for cat, house dust mite and pollen allergy, even though the precise mechanism of action is not yet clear (O'Hehir et al., 2016). T cell epitopes induce anergy or deletion of specific T cells, resulting in down-regulation of T cell proliferative and cytokine responses to the allergens, as in whole allergen IT (see Fig. 1.4, which summarises the main immunological events in the oral desensitization to food allergens), although the peptides may be too small to induce IgG-blocking antibodies (Prickett et al., 2015). Furthermore, also similarly to conventional IT (Fig. 1.4), an important role of T reg cells, with an increased production of IL-10 -that further induces the expansion of T reg cells and the inhibition of Th1, Th2 and inflammatory cells- has been reported (O'Hehir et al., 2016). Interestingly, cross-reactive T cell epitopes have been identified and, in some cases, tolerance to one T cell epitope is found to protect against other epitopes of the same molecule, providing evidence for the induction of linked epitope suppression (Moldaver and Larché, 2011). For its part, B cell epitope IT was proved to target different immune mechanisms aiding at pollen desensitization (Valenta et al., 2016). B cell epitopes prevent activation of mast cells and basophils, boost of memory IgE production and IgE-facilitated presentation to T cells, through the induction of IgG4 (Akdis and Akdis, 2014; Jongejan and van Ree, 2014). In the case of food allergies, IT with peptides has almost exclusively been assayed *in vivo* in murine models.

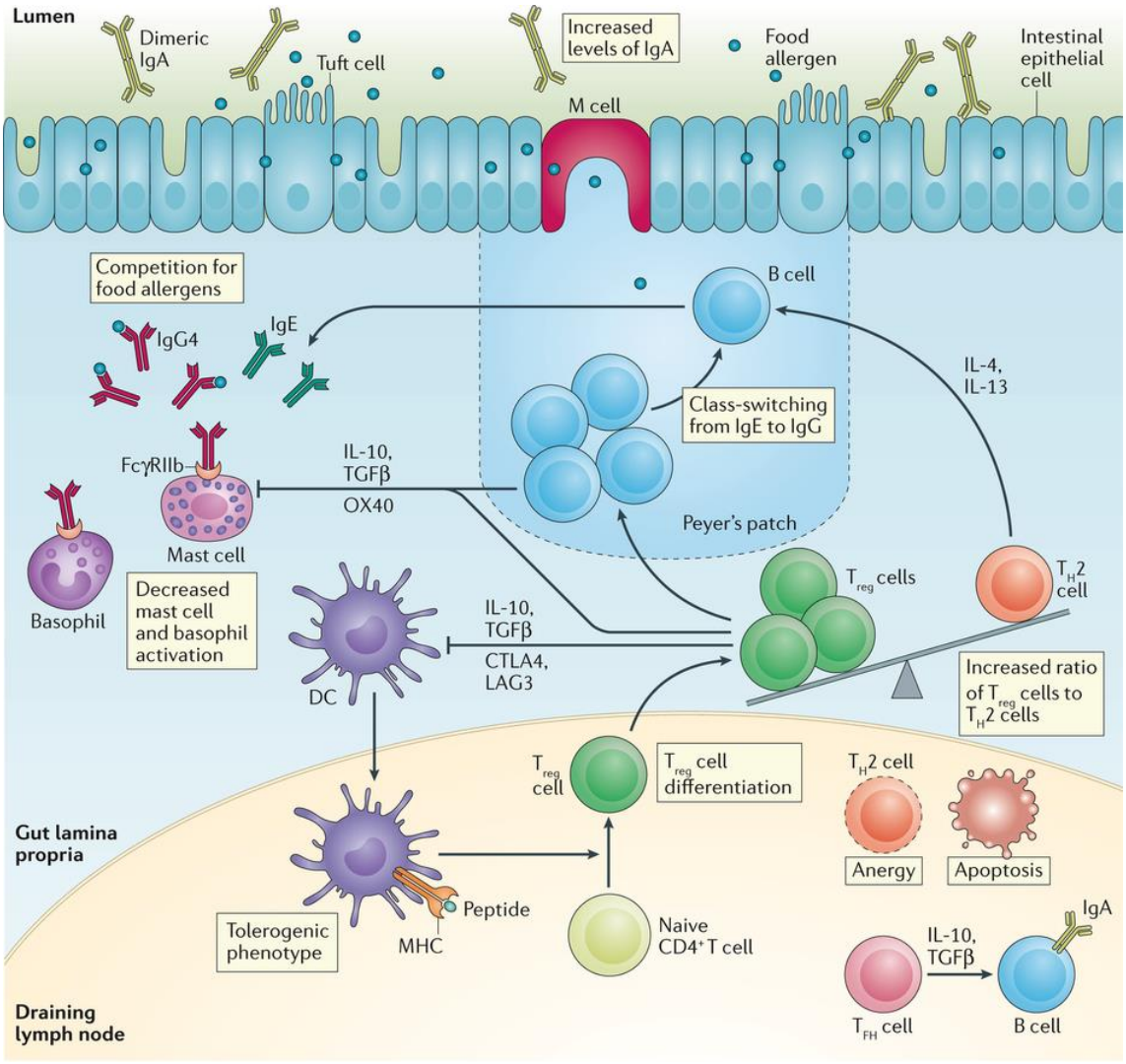

Figure 1.4. Mechanisms of desensitization to food allergens. Initially during desensitization, a shift occurs from the predominance of Th2 cells to that of allergen-specific T reg cells, which in turn may lead to the observed shift in allergen-specific antibodies from the IgE to the IgG4 isotype. T reg cells inhibit DCs, which is probably mediated by CTLA4 and LAG, and mast cells, via contact involving OX40–OX40L interaction. CTLA4, cytotoxic T lymphocyte antigen; DC, dendritic cell; FcγRIIb, inhibitory low-affinity IgG receptor type IIb; Ig, immunoglobulin; IL, interleukin; LAG3, lymphocyte activation gene 3; MHC, major histocompatibility complex; OX40, (CD134) member 4 of the tumour necrosis factor receptor superfamily; OX40L, (CD252) ligand for CD134; Tfh, T-follicular helper cell; Th, T-helper cell; T reg, regulatory T cell (reprinted with permission from Nature Publishing Group. Yu et al., 2016).

1.6. *In vivo* immunotherapeutic assays with food peptides

The results of a double-blind placebo-controlled randomized IT study with a hypoallergenic hydrolysate of whole egg white in egg allergic children have been recently published by Giavi et al. (2016). A significant increase in egg-specific IgG4 and a decreased

reactivity of basophils, usually associated with desensitization, was detected in the group of patients treated with the hydrolysate. The treatment, with few side effects, could be safely taken at home. However, the lack of statistically significant differences upon the final oral food challenge between the treatment and placebo groups suggests that further studies with a larger number of patients are needed to validate this therapeutic strategy and reveal the underlying mechanisms. To the best of our knowledge, this is the only published human trial with hydrolysates or peptides as IT for food allergy. For the meantime, promising findings have been obtained from studies using animal models of food allergy.

Mainly due to ethical reasons, animal models have become valuable tools to increase our understanding of the complex immunological and pathophysiological mechanisms involved in the development of food allergy (Finkelman, 2007; Van Gramberg et al., 2013). Such models allow the identification of factors responsible for the breakdown of oral tolerance and the implementation of experimental studies to establish prevention strategies and curative approaches, as well as to investigate novel therapies (Oyoshi et al., 2014). Rodents are frequently used in the field of the food allergy because of their small size, short breeding cycle, well characterized immunology and ability to product protein-specific IgE; in particular, murine strains, with or without genetic modifications, such as BALB/c, C3H/HeJ, DBA/2, A/J, BDF-1 and C57B1/6 and Brown Norway rats (Fritsché, 2009; Ladics et al., 2010).

As already mentioned, a pre-requisite of food peptides for being good candidates for IT is the lack of IgE reactivity and, consequently, of anaphylactic potential. *In vivo* assessments of the allergenicity of food protein hydrolysates, mainly hydrolyzed milk proteins, have been conducted by passive cutaneous anaphylaxis (PCA) tests, which measure the extravasation of a dye into the tissues after intravenous challenge of sensitized rodents or naïve rodents that previously received an intradermal injection of sera from sensitized ones (López-Expósito et al., 2012; Bøgh et al., 2015). Acute allergic skin responses -ear swelling- and local release of mouse mast cell protease-1 (mMCP-1)- can also be measured in sensitized mice following intradermal injection of allergen hydrolysates (van Esch et al., 2010). Furthermore, graded oral

administration of hydrolyzed and intact proteins to mice sensitized to the intact allergen allows comparing the doses that induce allergic symptoms and reduce body temperature (Kulis et al., 2012).

Table 1.2 summarises the main outcomes of the studies evaluating food protein derived hydrolysates and synthetic peptides for the treatment of food allergy in mice sensitized to the intact food allergens. In general terms, 2-4 weekly doses were administered, either orally or intraperitoneally, for 1 to 4 weeks. Many of the listed IT treatments were found to decrease adverse reactions upon subsequent challenge with the allergen, as measured by symptom scores and/or body temperature drops, in parallel with reductions in serum histamine release (Yang et al., 2010; Rupa and Mine, 2012) or mMCP-1 (Wai et al., 2016), that denote reduced activation and degranulation of mast cells. However, none of these studies re-assessed protection from anaphylaxis after a sufficiently extended period of discontinuation of the treatment and, therefore, sustained unresponsiveness (Pesek and Jones, 2016) cannot be guaranteed.

As shown in Table 1.2, desensitization with immunomodulatory hydrolysates and epitope peptides usually correlates with a significant reduction in allergen-specific IgE levels. However, the role of other allergen-specific antibodies, such as IgG and IgA, in mouse models, is under debate. Kulis et al. (2012) associated successful IT in the absence of changes in the IgE levels with significant increases in allergen-specific IgG1 and IgG2a, which would act as protective blocking antibodies. However, in other studies, no significant variations in allergen-specific total IgG levels were detected in the effectively treated mouse groups (Yang et al., 2009b and 2010), although, on further examination, alleviation of allergy signs was found to correlate with reduced IgG1 and increased IgG2a (Rupa and Mine, 2012). Wai et al. (2016) referred reductions in specific IgE, no change in specific IgG1, and high levels of specific IgG2a whose blocking effect was verified *in vitro* and *in vivo*. In humans, many studies demonstrate that increases in specific IgG4 levels accompany clinical improvements (Akdis and Akdis, 2014).

Table 1.2. Summary of successful immunotherapy trials in mouse models of food allergy with peptides and hydrolysates from allergenic proteins

Peptide/hydrolysate	Experimental design	Main outcomes	Reference
Egg white (EW) hydrolysed with a food grade aminopeptidase from *Aspergillus* sp. (EWH)	*Animal model:* BALB/c mice *Sensitization:* EW (5 mg) + CT (10 μg) i.g. (2 weekly doses for 4 weeks) *IT:* EWH (5mg) i.g. (3 weekly doses for 3 weeks) *Challenge:* EW (20 mg) i.g.	↓Histamine release ↓EW-specific IgE ↓IL-4 and ↓ IFN-γ released by EW stimulated splenocytes ↓IL-4, ↓IL-13, ↓IFN-γ, ↓IL-12p40, ↑IL-8, ↑IL-5,↑Foxp3 and ↑TGF-β intestinal (ileum) gene expression	Yang et al., 2009b
Mixture of ovalbumin (OVA) immunodominant T cell epitopes (15-mer): AMVYLGAKDSTRTQ SWVESQTNGIIRNVL AAHAEINEAGREVVG	*Animal model:* BALB/c mice *Sensitization:* OVA (1 mg) + CT (10 μg) i.g. (2 weekly doses for 4 weeks) *IT:* peptide mix (100 μg each) s.c. (3 weekly doses for 3 weeks) *Challenge:* OVA (20 mg) i.g.	↓Symptom score ↓Histamine release ↓OVA-specific IgE ↑OVA-specific faecal IgA ↓IL-4 and ↑IFN-γ released by OVA stimulated splenocytes ↓IL-5, ↓IL-13, ↑IFN-γ, ↑Foxp3 and ↑TGF-β intestinal (ileum) gene expression	Yang et al., 2010
Ovomucoid (OM) immunodominant T cell epitope (15-mer): DNKTYGNKSNFSNAV	*Animal model:* BALB/c mice *Sensitization:* OM (1 mg) + CT (10 μg) i.g. (2 weekly doses for 4 weeks) *IT:* peptide (1 mg) i.g. (3 weekly doses for 4 weeks) *Challenge:* OM (20 mg) i.g.	↓Symptom score ↓Histamine release ↓OM-specific IgE, ↑IgG1,↓IgG2a, ↑faecal IgA ↓IL-4, ↑IL-12, ↑IL-10 released by OM stimulated splenocytes ↑circulating T reg cells (↑CD4+CD25+, ↑CD4+Foxp3+ cells)	Rupa and Mine, 2012
Native cashew protein (nCSH) hydrolysed with pepsin (pCSH)	*Animal model:* C3H/HeJ mice *Sensitization:* nCSH (2 mg) + CT (10 μg) i.g. (4 weekly doses for 4 weeks) *IT:* pCSH (from 50 to 200 μg) i.p. (3 weekly doses for 4 weeks) *Challenge:* nCSH (0.25 mg) i.p. or pCSH (1 mg) i.p	↓Symptom score and body temperature drop No change in nCSH specific IgE ↑nCSH-specific IgG1 ↑nCSH-specific IgG2a ↓IL-5 and ↓IL-13 released by nCSH stimulated splenocytes	Kulis et al., 2012
β-lactoglobulin (BLG) immunodominant T cell epitope AQKKIIAEKTKIPAVFKIDALN	*Animal model:* BALB/c mice *Sensitization:* BLG (50 μg) + alum i.p. (1 weekly dose for 3 weeks) *IT:* peptide (1 mg) i.p. (3 weekly doses for 4 weeks) *Challenge:* BLG (50 mg) i.g.	↓Symptom score and body temperature drop No change in BLG-specific IgE, IgG1, IgG2 or faecal IgA ↑IFN-γ, ↑IL-12, ↑IL-10 released by BLG stimulated splenocytes	Thang and Zhao, 2015

Mixture of 6 shrimp tropomyosin (Met e 1) immunodominant T cell epitopes	*Animal model:* BALB/c mice *Sensitization:* recombinant Met e 1 (0.1 mg) + CT (10 μg) i.g. (1 weekly dose for 5 weeks) *IT:* peptide mix (1.2 mg each) i.g. (2 weekly doses for 4 weeks) *Challenge:* recombinant Met e 1 (0.5 mg) i.g.	↓Symptom score, diarrhoea and intestinal inflammation ↓mMCP-1 ↓Met e 1-specific IgE ↑Met e 1-specific IgG2a ↓IL-4, ↓IL-5, ↑IFN-γ, ↑IL-12 released by Met e1 stimulated splenocytes ↑IL-10, ↑Foxp3, ↑CD25, ↑RUNX1, ↑RUNX3, ↓IL-5, ↓IL-13, ↑FN-γ intestinal (ileum) gene expression	Wai et al., 2016

Experimental design: i.g.= intragastric; i.p..= intraperitoneal; s.c.= subcutaneous; CT= cholera toxin. **Main outcomes:** mMCP-1= mouse mast cell protease 1; RUNX1= Runt-related transcription factor 1; RUNX3= Runt-related transcription factor 3;↑= increased or high levels; ↓= decreased or low levels

However, homology between mouse and human IgG subclasses is difficult to stablish, and there are differences in class switching: in mice, IL-4 induces IgG1 and IgE, whereas in humans, IL-4 induces switching to IgG4 and IgE (Mestas and Hughes, 2004; Finkelman, et al., 2016).

Circulating IgG antibodies can neutralize allergens before they cross-link mast cell-bound IgE, although this ability to physically inhibit the binding of allergens to IgE is saturable and depends on the challenge dose (Finkelman, 2005). Furthermore, it has been described that anaphylaxis in the mouse can occur through two independent pathways, comprising either the classical mechanism associated with human allergy -that is the cross-linking of IgE bound to FcεRI on mast cells- or a second, alternative, pathway that requires IgG antibodies, FcγRIII receptors and macrophages (see Fig. 1.5, which outlines the mechanisms of anaphylaxis mediated by basophils, mast cells and macrophages in mice). Both share similar time courses and symptoms, but much higher antibody and antigen concentrations are required to induce anaphylaxis by the alternative IgG-mediated pathway, in line with the much higher affinity of FcεRI for IgE than FcγRIII for IgG and with the need for antigen-IgG complexes to form in blood before they bind FcγRIII on macrophages (Finkelman, 2007). IgE-mediated anaphylaxis predominates in the presence of low levels of IgG and allergen, even if the IgE levels are also low.

In some mouse models of allergy, sensitization stimulates high levels of specific IgG and mice are challenged with large quantities of allergen, which could inhibit IgE-mediated anaphylaxis, but also induce IgG-mediated anaphylaxis (Strait et al., 2006). This implies that the protective effect of blocking IgG antibodies could be restricted to a situation when the quantity of challenge allergen is less than that required to trigger IgG-mediated anaphylaxis; but when the dose increases, IgG or both IgG and IgE pathways could be triggered (Fig. 1.5) (Strait et al., 2006). Thus, the outcomes of different IT experiments with respect to changes in relative antibody levels may not be comparable, due to the use of different immunization protocols that produce different ratios of IgE to IgG and different amounts of allergen for challenge, particularly if antibody analyses are performed short after challenge. Furthermore, the IgE/IgG

response may also vary depending on the administration route (oral, subcutaneous or intraperitoneal) of the sensitizing allergens and IT preparations (Finkelman et al., 2005; Kulis et al., 2012).

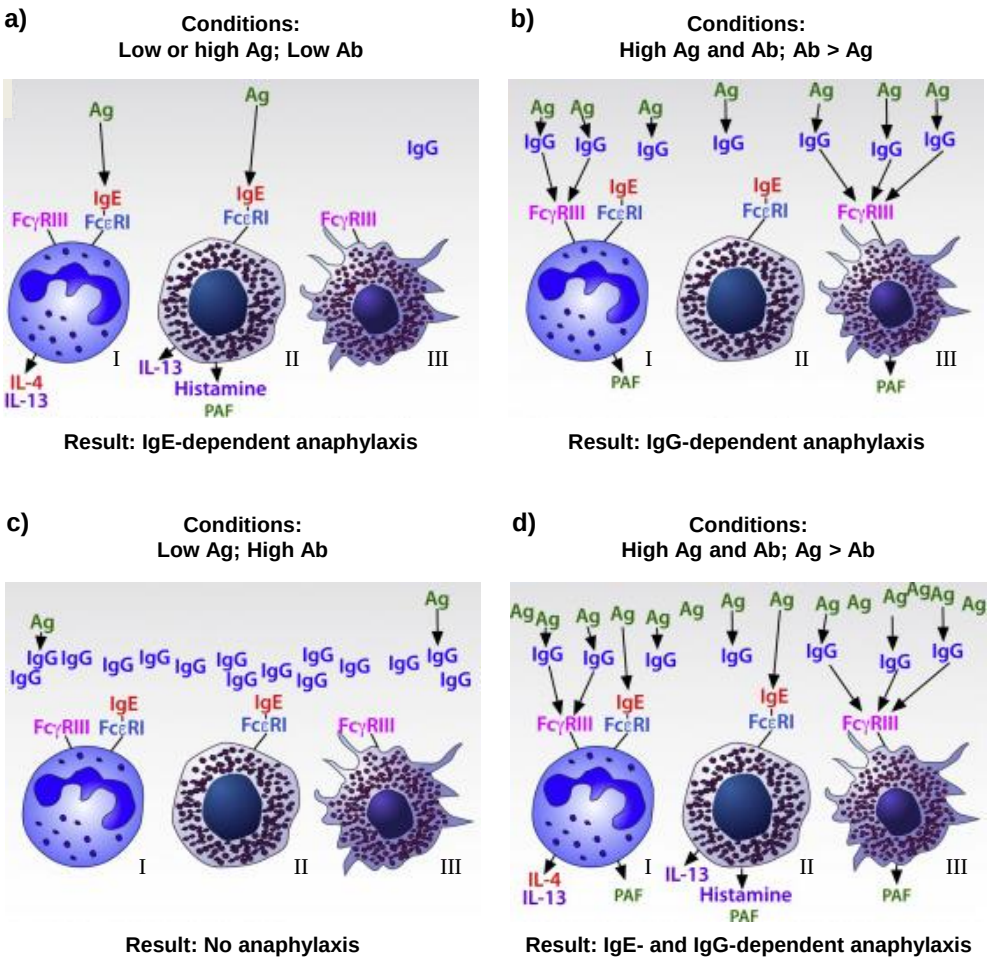

Figure 1.5. The role of antigen (Ag)-specific IgE and IgG antibodies (Ab) in anaphylaxis. When Ab levels are low (a), only IgE-mediated anaphylaxis can occur. When Ag and Ab levels are both high but Ab levels are in excess to Ag levels (b), IgGs block the binding of Ag to FcεRI-bound IgE, but IgG/Ag complexes can bind to FcγRs; consequently, only IgG-mediated anaphylaxis occurs. When Ag levels are low but Ab levels are high (c), IgG "blocking" Abs prevent IgE-mediated anaphylaxis by intercepting Ag before it can bind to FcεRI-associated IgE and by binding to the inhibitory receptor FcγRIIB, but the quantity of IgG/Ag complexes is too low to trigger IgG-mediated anaphylaxis; consequently, anaphylaxis does not occur. When Ag and Ab levels are both high but Ag levels are in excess (d), IgG/Ag complexes are sufficient to trigger IgG-mediated anaphylaxis, and enough Ag escapes IgG blockade to bind to FcεRI-associated IgE and trigger IgE-mediated anaphylaxis. FcγRIII, stimulatory low-affinity IgG receptor type III; FcγRIIb, inhibitory low-affinity IgG receptor type IIb; FcεRI, high-affinity IgE receptor type I; Ig, immunoglobulin; IL, interleukin; PAF, platelet-activating factor; I, basophil; II, mast cell; III, macrophage (reprinted with permission from Elsevier. Finkelman et al., 2016).

Secretory IgA has an important role in immune homeostasis in the gut, with low levels of intestinal allergen-specific IgA being associated with the development of food allergy, although its role is still unclear (Kukkonen et al., 2010). Following some effective IT studies with food peptides, increased levels of fecal allergen-specific IgA have been reported (Yang et al., 2010; Rupa and Mine, 2012). However, the hypothesis that IgA secreted at mucosal surfaces can bind allergens before they are absorbed, in a way similar to its neutralizing effect towards pathogens and toxins, could not be substantiated. Instead, the property of IgA to block systemic anaphylaxis by binding to allergens appears to be restricted to serum IgA and not to IgA contained in the gut lumen (Strait et al., 2011).

With respect to cellular responses, a consistent reduction in the production of Th2 cytokines (whether IL-4, IL-5 or IL-13) was observed when splenocytes from treated animals were stimulated with the whole allergens (Table 1.2). However, the reports refer either an associated increase in Th1 cytokines (IFN-γ or IL-12) and restoration of the immunological balance (Yang et al., 2010; Rupa and Mine, 2012; Wai et al., 2016), or a concomitant Th1 decrease (Yang et al., 2009b). Ileum expression levels of Th2 and Th1 cytokine genes parallel systemic changes in the Th2 and Th1 cytokine profiles (Yang et al., 2009b; Yang et al., 2010; Wai et al., 2016). Upregulation of TGF-β and IL-10 or of the transcription factor Foxp3 in intestinal tissues points at a role of T reg cells in tolerance induction by food peptides (Yang et al., 2009b and 2010; Wei et al., 2016). Increased percentages of T reg cells (CD4+ Foxp3+ and CD4+ CD25+ cells) were also detected in the blood of the desensitized animals (Rupa and Mine, 2012).

While the administration of multiple T cell epitopes appears more effective than that of the corresponding individual peptides (Yang et al., 2010), it is noteworthy that a single immunodominant T cell peptide can offer protection against challenge with the whole allergen, which reinforces the concept of linked epitope suppression (Table 1.2). In this respect, it is speculated that the only effective way to provide tolerance towards multiple epitopes within a protein -or even towards other allergens during co-exposure- by the use of a single peptide is

through the induction of a regulatory function versus a delectional function, which may depend on the choice of route, dose and timing of administration of IT (Mackenzie et al., 2012).

Decreased allergic symptoms following IT were induced by strong Th2-stimulating hydrolysates, which maintain the immunogenicity of the intact allergen and its sensitizing potential, but lack eliciting potential (Kulis et al., 2012). In any case, immunomodulating peptides, selected on the basis of their strong proliferative activity, IFN-γ stimulating properties and absence of IL-4 inducing capacity, stand as an interesting option (Yang et al., 2010; Wei et al., 2016). Nonetheless, the observation that different peptides that reduce allergen-induced clinical signs produce different immunological changes highlights the need for a deeper knowledge on the structure-function relationships to go deeper into their mechanism of action (Yang et al., 2010; Rupa and Mine, 2012).

1.7. *In vivo* allergy prevention assays with food peptides

A recognized advantage of IT lays in its potential to restrain the progression of mild to severe forms of allergy. This suggests its use in sensitized individuals to avoid the development of allergic symptoms or, even as a prior strategy, to prevent sensitization (Valenta et al., 2016). In fact, it has been shown that, rather than their avoidance, the early introduction of eggs or peanuts in the diet significantly decreases the frequency of egg or peanut allergy among genetically predisposed children, which, in both cases, correlates with increased levels of peanut-specific IgG4 (Palmer et al., 2013; Du Toit et al., 2015). Furthermore, prophylactic house dust mite therapy in children at high heredity risk was demonstrated to reduce sensitization to common allergens (Zolkipli et al., 2015). However, as already indicated for curative treatments of food allergy, the use of intact allergens for preventive treatments poses a risk of anaphylactic reactions and it does not fully exclude the possibility of a progress in the development of sensitization. Once more, peptides or hydrolysates appear as an attractive alternative, but it is necessary to assure that they lack sensitizing potential.

The sensitizing capacity of food hydrolysates can be evaluated in Brown Norway rats immunized by the intraperitoneal route without adjuvant, by analyses of specific antibody responses. Induction of specific IgE, IgG1 and IgG2a is taken as a measure of sensitization, together with the demonstration of the biological activity of the generated IgE in basophil activation assays (Bøgh et al. 2009 and 2013; Kroghsbo et al., 2014). Release of rat mast cell protease II (RMCPII) indicates IgE-mediated mucosal mast cell degranulation (Fritsché et al., 1997). The use of aluminum hydroxide as adjuvant enhances immunogenicity and narrows heterogeneity between experimental data, but may bias the results by changing antigen presentation to the immune system (Bøgh et al., 2015). On the other hand, the oral route, more physiologically relevant, gives comparable outcomes, but with different dose-response ratios, with respect to the intraperitoneal route (Kroghsbo et al., 2014).

Mice have also been used as models of food allergy for the evaluation of the sensitizing capacity of hydrolyzed proteins. Sensitization of mice to food proteins requires the use of Th2-polarizing adjuvants -such as staphylococcal enterotoxin B and, more commonly, cholera toxin (CT)- to overcome their strong tendency to develop oral tolerance, although this practice may conceal the intrinsic sensitizing properties of the co-administered proteins (Berin and Shreffler, 2008). In C3H/HeOuJ mice, comparison between the levels of allergen-specific IgE, IgG1 and IgG2a, induced by hydrolysed and intact allergens administered orally with CT is used for the assessment of lack of sensitizing potential of the formers, which can be confirmed by subsequent challenge with the whole allergen and measurement of anaphylactic symptoms, body temperature and mMCP-1 (van Esch et al. 2011a). An inter-laboratory evaluation of this model in four different research centers evidenced its transferability and adequacy to the prediction of the residual sensitization capacity of hydrolysed milk-based infant formulas (van Esch et al., 2013), which, according to European guidelines on hypoallergenic and follow-on infant formulas, should not be able to sensitize animals to the protein source they derive from (Commission Directive 96/4/EC).

Most interventions aimed at inducing protective responses against food allergy have been conducted with infant hypoallergenic formulas derived from enzymatic hydrolysis of cow's milk proteins. These have concluded that certain hydrolyzed infant formulas, in particular partially hydrolyzed whey formulas, have a role in the reduction of risk of atopic disease (Szajewska and Horvath, 2010; Vandenplas et al., 2014). Further evidence for the efficacy and mechanisms of action of protein hydrolysates in allergy prevention were obtained from mice trials, as shown in Table 1.3.

Administration of intact and hydrolyzed milk proteins avoid subsequent sensitization, as measured by inhibition of the generation of allergen-specific IgE, release of mast cell mediators and allergic symptoms (Table 1.3). However, in certain studies, only the intact proteins were able to significantly reduce serum levels of specific IgE and/or IgG1, indicating a more effective prevention of sensitization (Adel-Patient et al., 2011 and 2012; van Esch et al., 2011b). In any case, protection against the development of an allergic response can also be induced by specific peptides present in partially hydrolyzed preparations but absent from extensively hydrolysed formulas (Fritsché et al., 1997; van Esch et al., 2011b), although more prolonged treatments may be necessary to achieve effects similar than those of the intact allergens (Peng et al., 2004). In addition, the effect is dose-dependent, with peptide enrichment decreasing the efficient oral dose, although correlation between peptide structure and tolerogenic properties has not been described (Pecquet et al., 2000; Hacini-Rachinel et al., 2014).

Simultaneous immunization of rats with intact β-lactoglobulin plus hydrolysed β-lactoglobulin reduces the sensitizing capacity of the former, which was attributed to a tolerogenic affect exerted by the digestion products (Bøgh et al. 2013). A single peptide from β-lactoglobulin (β-lactoglobulin 31-48) was reported to reduce sensitization and allergic response to the whole intact protein in CeH/HeOuJ mice, thus inducing linked epitope suppression, with stronger effects on its own than when present in a peptide mixture (Meulenbroek et al., 2013). However, the peptide β-lactoglobulin 25-107, which represents 50% of the protein sequence and comprises the former, was not found to decrease further sensitization in BALB/c, despite it

Table 1.3. Summary of successful preventive treatment trials in mouse models of food allergy with peptides and hydrolysates from allergenic proteins

Peptide/ hydrolysate	Experimental design	Main outcomes	Reference
Standard formula of intact cow's milk proteins Whey proteins (WP) partially hydrolysed with trypsin	*Animal model:* Sprague-Dawley rats *Preventive treatment:* Oral administration in the drinking water during 19 days (*ad libitum*) *Sensitization:* β-lactoglobulin (BLG) (100 µg) + AH i.p. (1 dose) *Challenge:* WP (1 g) i.g.	↓RMCPII ↓BLG-specific IgE ↓BLG-specific IgG ↓Lymphocyte proliferation by BLG-stimulated PLN cells	Fritsché et al., 1997
β-lactoglobulin (BLG) BLG hydrolysed with trypsin (TTH-BLG) Peptide fractions separated from TTH-BLG by ion exchange chromatography	*Animal model:* BALB/c mice *Preventive treatment:* BLG (5 mg/g body weight), TTH-BLG (2.5 mg/g) or peptide fractions (0.125 mg/g) i.g. *Sensitization:* BLG (80 µg) + AH (1 dose) *Challenge:* BLG (100 µg) i.d. in the footpad	↓Footpad thickness ↓BLG-specific IgE ↓Lymphocyte proliferation by BLG-stimulated splenocytes	Pecquet et al., 2000
Standard formula of intact cow's milk proteins (CM, Nan) CM partially hydrolysed (Nan-HA)	*Animal model:* C3H/HeN mice *Preventive treatment:* Oral administration at 2% (1 to 4 weeks) *Sensitization:* β-lactoglobulin (BLG), α-lactalbumin (ALA) or BSA (10 µg) + alum i.p. (1 dose)	↓BLG, ALA and BSA-specific IgE (1 week for Nan, 4 weeks for Nan-HA) ↓BLG, ALA and BSA -specific IgG (1 week for Nan, 4 weeks for Nan-HA) ↓BLG ALA and BSA, specific IgG1 (1 week for Nan, 4 weeks for Nan-HA)	Peng et al., 2004
Whey proteins (WP) Partially hydrolysed WP (pWH)	*Animal model:* C3H/HeOuJ mice *Preventive treatment:* WP and pWH (50 mg) i.g. (1 daily dose for 6 days) *Sensitization:* WP (20 mg) + CT (10 µg) i.g. (1 weekly dose for 6 weeks) *Challenge:* WP (10 µg) i.d.	↓Acute skin response ↓mMCP-1 ↓WP-specific IgE and IgG1 (WP) ↑MLN T reg cells (pWH), ↑MLN Th1 (WP), ↓MLN Th2 (pWH and WP)	van Esch et al., 2011b
β-lactoglobulin (BLG) BLG hydrolysed with trypsin (BLG-Try)	*Animal model:* BALB/c mice *Preventive treatment:* BLG (0.05-4 mg) and BLG-Try (2 mg) i.g. (6 doses in 10 days) *Sensitization:* BLG (5 µg) + alum i.p. (2 doses in 14-18 days) *Challenge:* BLG (10 mg) i.g.	↓mMCP-1 (BLG) ↓BLG-specific IgE ↓BLG-specific IgG1: (BLG) ↓IL-4, ↓IL-5 ↓IL-10 ↓IL-12 ↓IL-17 ↓IL-5 released by BLG stimulated splenocytes (BLG) ↑MLN T reg cells (↑ CD4+CD25+Foxp3+ cells) ↑PP and spleen T reg cells (↑ CD4+CD25+Foxp3+ cells) (BLG)	Adel-Patient et al., 2011

Native β-lactoglobulin (nBLG) Denatured BLG (dBLG) nBLG hydrolysed with CNBr (nBLG-CBr) dBLG hydrolysed with CNBr (dBLG-CBr)	*Animal model:* BALB/c mice *Preventive treatment:* nBLG, dBLG, nBLG-CNBr and dBLG-CNBr (2.4 mg) i.g. (6 doses in 10 days) *Sensitization:* nBLG (5 µg) + alum i.p. (1 dose)	↓BLG-specific IgE ↓BLG-specific IgG1 ↓IL-4, ↓IL-5, ↓IL-13, ↓IL-10 released by BLG stimulated splenocytes	Adel-Patient et al., 2012
β-lactoglobulin (BLG) immunodominant T cell epitopes: BLG 31-48 Mix 1: 13-30, 19-36, 25-42, 31-48 Mix 3: 139-156, 145-162	*Animal model:* CeH/HeOuJ mice *Preventive treatment:* peptide mix or single peptide (4 mg each) i.g. (1 daily dose during 6 days) *Sensitization:* WP (20 mg) + CT (10 µg) i.g. (1 weekly dose for 5 weeks) *Challenge:* WP (10 µg) i.d. and WP (100 mg) i.g.	↓ Acute skin response ↓ WP-specific IgE ↓ WP-specific IgG2a ↑ MLN T reg cells (↑ CD4+CD25+Foxp3+ cells) (BLG 31-48) ↑ MLN DCs (CD11b+CD103+) (BLG 31-48)	Meulenbroek et al., 2013
Whole hen's egg (WE) WE hydrolysed with Protamex and Flavourzyme 1000L (HE)	*Animal model:* C57BL/6J mice *Preventive treatment:* Oral administration of OVA, WE, and HE (5 times more concentrated) in the drinking water during 5 days (*ad libitum*) *Sensitization:* Ovalbumin (OVA) (100 µg) + alum (2 doses in 13 days) s.c. *Challenge:* OVA (20 µg) i.d.	↓Acute skin response ↓OVA-specific IgE ↓OVA-specific IgG1 ↓Proliferation of OVA stimulated splenocytes ↓IL-5, ↓IL-4, ↓IFN-γ, ↓IL-10, IL-12 released by OVA stimulated splenocytes ↑MLN T reg cells (↑ CD4+CD25+Foxp3+ICOS+cells)	Hacini-Rachinel et al., 2014
Partially hydrolysed whey proteins (pWP)	*Animal model:* C3H/HeOuJ *Preventive treatment:* pWP (50 mg) i.g. (1 daily dose for 5 days) *Sensitization:* WP (20 mg) + CT (10 µg) i.g. (1 weekly dose for 6 weeks) *Challenge:* WP (10 µg) i.d. and WP (50 mg) i.g.	↓Acute skin response and symptoms score ↓WP-specific IgE	Van den Elsen et al. 2015

Peptide/hydrolysate: CNBr=cyanogen bromide. **Experimental design:** i.g.= intragastric; i.d.= intradermal; i.p.= intraperitoneal; s.c.= subcutaneous; CT= cholera toxin, AH= aluminium hydroxide. **Main outcomes:** mMCP-1= mouse mast cell protease 1; RMCPII= rat mast cell protease II; MLN= mesenteric lymph nodes; PLN= peripheral lymph nodes; PPs = Peyer's patches; DCs= dendritic cells; ↑= increased or high levels; ↓= decreased or low levels

exerts immunostimulating properties in splenocytes from β-lactoglobulin sensitized mice (Adel-Patient et al., 2012).

Preventive approaches using hydrolysates or peptides lead to reduced numbers of activated Th2 cells in the MLNs, following sensitization and allergen challenge of mice (van Esch et al., 2011b), as well as to decreased systemic Th2 responses, as shown by a diminished release of IL-4, IL-5 and IL-13- upon stimulation of spleen cells with the allergens used for sensitization (Adel-Patient et al., 2012; Hacini-Rachinel et al., 2014). Changes in the Th1 response are, however, less consistent among the studies (Table 1.3). Combination of peptide treatment with a prebiotic diet that skews the immune response to a Th1 response may increase its tolerance-driving capacity (Meulenbroek et al., 2013). Conversely, co-administration of omega-6 polyunsaturated fatty acid-rich soya oil, which increases the Th2/Th1 ratio, abrogates peptide-induced tolerance (van den Elsen et al., 2015).

In addition, some studies showed a key role of T reg cells induced at the MLN level in the prevention of sensitization and development of food allergy (Adel-Patient et al., 2011; van Esch et al., 2011b; Meulenbroek et al., 2013; Hacini-Rachinel et al., 2014). MLNs connect the local and systemic immune system, making it possible to translate gut-induced regulatory responses to systemic tolerance. Indeed, adoptive transfer of MLN cells from tolerized donor mice inhibits sensitization and reduces consequent anaphylactic effects in recipient mice (van Esch et al., 2011b). The generation of a regulatory response by peptides is supported by a correlation between T reg cells and augmented tolerogenic CD103+ DCs (Meulenbroek et al., 2013).

Progress in the understanding of the stimulating and anti-inflammatory properties of food peptides on cells of the innate and adaptive immune system has made apparent that they represent an attractive means to enhance safety and efficacy of IT against food allergy. Moreover, the conclusions drawn from *in vivo* curative and preventive trials are promising, although there is a need for more studies to further explore the immunomodulating strategy and

its mechanisms to maximise effective clinical translation. In particular, knowledge of the peptide sequence and structural requirements which determine the immunomodulatory action depending on the cell type, currently still poorly understood, will open new possibilities to impact the outcome of IT by differentially influencing the mechanisms by which oral tolerance to allergens is stablished.

2. OBJECTIVE AND WORK PLAN

Egg allergy is among the most common food allergies in European children below the age of three, with a prevalence ranging between 0.2 and 2%, as judged by objective appraisals (Nwaru et al., 2014). Despite many children spontaneously outgrow this condition, up to 15-20% remains allergic and, within the population with persistent egg allergy, the severity of the adverse reactions to egg usually increases over the years (Sicherer et al., 2014). The management of egg allergy is limited to strict avoidance of the offending food and emergency treatment of adverse reactions in case of accidental exposure. However, total avoidance is exceedingly difficult from a practical standpoint since egg and egg-derived products are ubiquitous ingredients (Martorell et al., 2013; Wood, 2016).

OIT using whole egg white (EW) is one of the most promising treatment options for egg allergy (Vickery, 2012). This type of OIT has shown to be efficient in the induction of desensitization, with a rate of success between 50-90% (Iacono et al., 2013; Meglio et al., 2013; Perezábad et al., 2015). However, a major drawback of OIT using intact egg allergens is the high risk of severe side effects, which has prompted the investigation of strategies aimed at reducing the allergenicity of egg-derived preparations used as treatment (heat treatment, fermentation, genetic modification, sugar conjugation and enzymatic hydrolysis). These preparations have to maintain or improve their immunomodulating potential to achieve effective desensitization or sustained oral tolerance (O'Hehir et al., 2016; Pesek and Jones, 2016; Vázquez-Ortiz and Turner, 2016).

Among the most promising strategies currently assayed against IgE-mediated allergic diseases stands the possibility of using immunomodulating peptides to stimulate oral tolerance towards offending food allergens or even to prevent allergic sensitization (Yang et al., 2010; Casale and Stokes, 2011; Moldaver and Larché, 2011). In this framework, enzymatically digested allergens could be a more practical therapeutic option than synthetic dominant T cell epitopes, as the former would provide multiple allergen-derived peptides with adequate T cell-stimulating capacities for HLA-diverse patients (Yang et al., 2009b; Kulis et al., 2012). Thus, hydrolysis of the major egg white allergens, ovomucoid (OM -*Gal d 1*-), ovalbumin (OVA -*Gal*

d 2-) and lysozyme (LYS -*Gal d 4*-), appears as an attractive and safe alternative for a reproducible and standardized production of immune-active peptides with low costs that would allow their consumption in the form of functional foods.

Given the importance of immunologically active peptides in food allergy, **the aim of this thesis** was the development of enzymatic hydrolysates of egg white proteins with hypoallergenic and immunomodulating properties that could be used in the prevention and treatment of egg allergy. For this purpose, we addressed the preparation and characterization of various hydrolysates and their evaluation using *in vitro* and *ex vivo* model systems of increasing complexity for assessing their potential in the regulation of immune and inflammatory processes typical of allergic diseases. Finally, we confirmed the efficacy of selected hydrolysates in an *in vivo* model of egg allergy.

The first step was the preparation and characterization of a collection of enzymatic hydrolysates from OVA, LYS, OM and EW with pepsin, Neutrase and alcalase. The potential effects of these hydrolysates on oxidative stress, cell proliferation and modulation of immune responses were then evaluated on cultures of murine spleen and MLN cells unspecifically stimulated with T or B cell mitogens (Fig. 2.1).

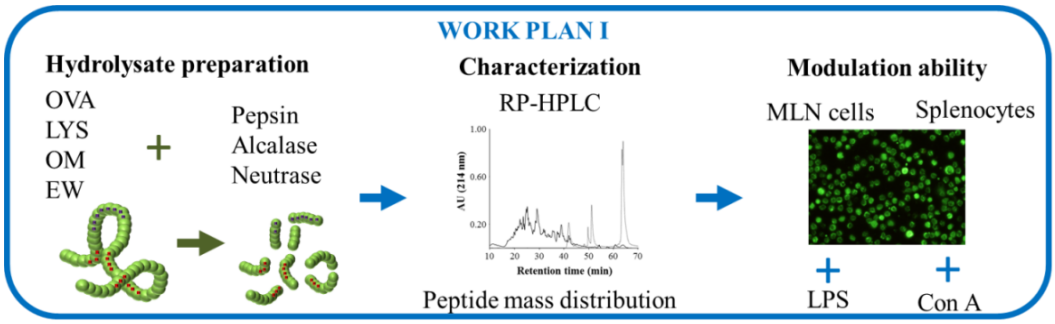

Figure 2.1. Work plan I for the preparation and characterization of hydrolysates of egg white proteins and the study of their ability to modulate immune responses in unspecifically stimulated murine cells. Con A, concanavalin A; EW, whole egg white; LPS, lipopolysaccharide; LYS, lysozyme; MLN, mesenteric lymph nodes; OM, ovomucoid; OVA, ovalbumin; RP-HPLC, reverse phase high-performance liquid chromatography.

Once evidenced that the hydrolysates could drive immune responses in a certain direction, we next assessed their ability to deviate the unbalanced immune status typical of food

allergy. Thus, the second stage was to evaluate the immunomodulating potential of the hydrolysates as their ability to hinder, on the one hand, cytokine and IgE production by Th2-skewed human PBMCs and, on the other hand, the release of pro-inflammatory factors and ROS generation from peripheral blood leukocytes (PBLs) subjected to a Th1 stimulus. The binding to IgE from egg allergic patients was also determined (Fig. 2.2.)

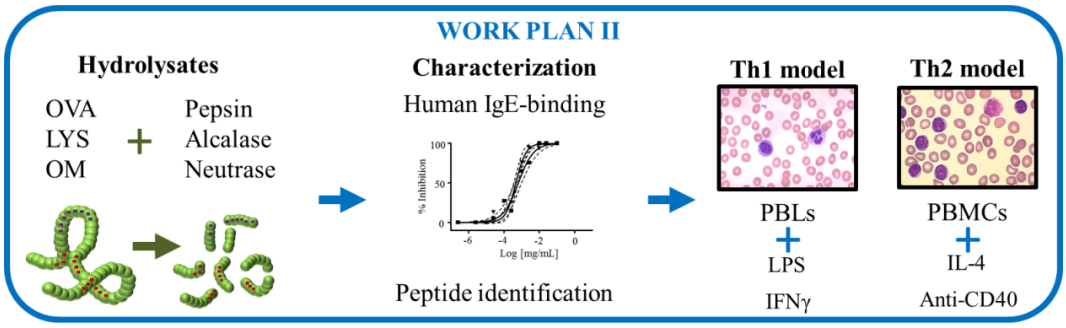

Figure 2.2. Work plan II for the study of the allergenicity of the hydrolysates and their ability to regulate exacerbated responses in Th1- and Th2-skewed human peripheral blood cells. . LPS, lipopolysaccharide; LYS, lysozyme; OM, ovomucoid; OVA, ovalbumin; PBLs, peripheral blood leukocytes; PBMCs, peripheral blood mononuclear cells; Th, T-helper cell.

It was shown that low IgE-binding hydrolysates could modify the cellular and humoral immune responses of human peripheral blood cells to different stimuli, suggesting potential activities that could be used to polarize Th2 and Th1 immune functions. Therefore, we next assessed the effects of the peptides contained in the hydrolysates in a specific *ex vivo* model of egg allergy. The ability of the hydrolysates to modulate T cell cytokine responses to egg allergens was evaluated in splenocytes from mice sensitized to individual egg proteins, or their mixture in different proportions, and their allergenicity *in vivo* was also estimated (Fig. 2.3). In addition, the hydrolysates were fractionated, in an attempt to enrich the immunostimulating and immunomodulating peptides, and the sequences contained in each fraction were identified by mass spectrometry.

Finally, the most promising hydrolysates were selected and their ability to modify beneficially the immune reaction to egg allergens was tested *in vivo* using a mouse model of egg allergy. As a first step, the lack of sensitizing and eliciting potential was evaluated, to then

determine the efficacy of the hydrolysates both as preventive and therapeutic agents. Special attention was paid to the elucidation of the mechanism of clinical protection.

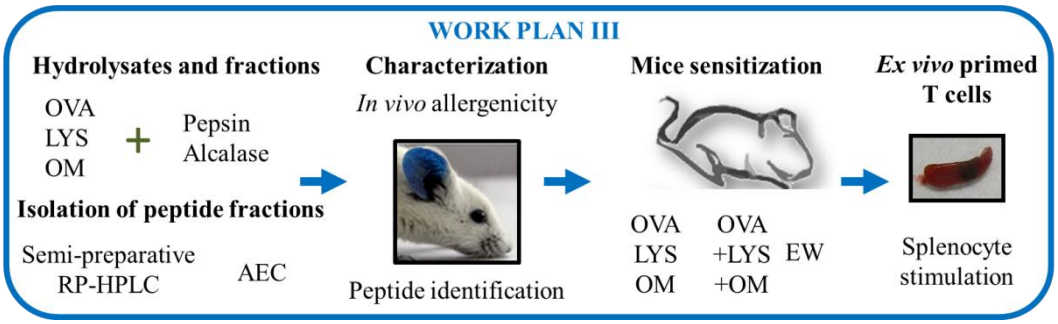

Figure 2.3. Work plan III for the *in vivo* assessment of the allergenicity of the hydrolysates and the study of their capacity to modulate allergen responses induced *ex vivo* on spleen cells from mice sensitized to egg white proteins. AEC, anion exchange chromatography; EW, whole egg white; LYS, lysozyme; OM, ovomucoid; OVA, ovalbumin; RP-HPLC, reverse phase high-performance liquid chromatography.

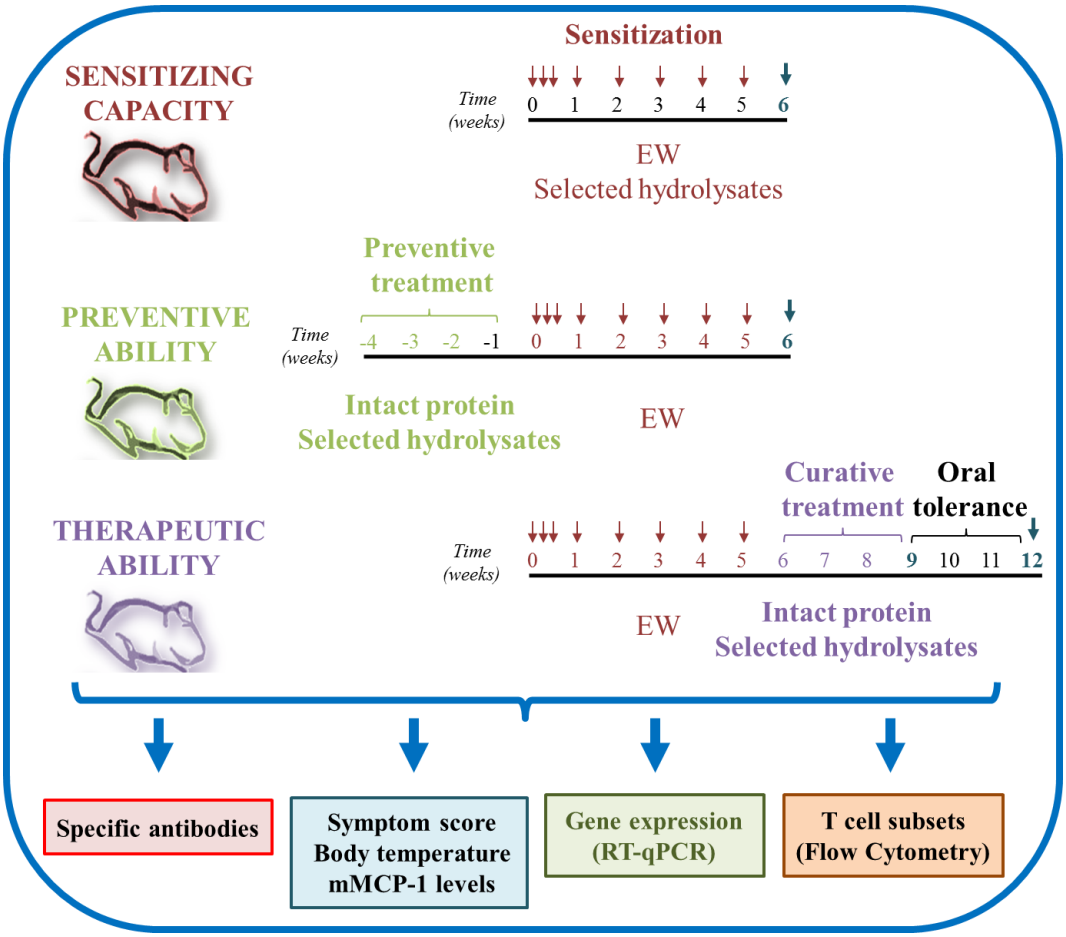

Figure 2.4. Work plan IV for the *in vivo* assessment of the sensitizing capacity of selected hydrolysates and the study of their ability to prevent and treat egg allergy. EW, whole egg white; mMCP-1, mouse mast cell protease 1; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

3. MATERIALS AND METHODS

3.1. Production of enzymatic hydrolysates from egg white proteins

OVA grade VI, LYS and OM were purchased from Sigma-Aldrich (St. Louis, MO, USA). EW was separated from fresh eggs, lyophilized, and its protein content was determined by the Kjeldahl method. LPS level of the proteins was quantified by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Waltham, MA, USA), according to the instructions of the manufacturer. The LPS contamination of LYS, OM and EW was lower than 1 UE/mg and they were used as such; while OVA was purified from LPS using size exclusion chromatography, as described by Pablos-Tanarro et al. (2016). This procedure reduced the LPS content of OVA from 445.8 UE/mg to 3 UE/mg.

Protein solutions (5 mg/mL) were prepared in either MilliQ water adjusted to pH 1.5 (for hydrolysis with pepsin) or in phosphate buffer pH 7.0 (for hydrolysis with Neutrase and alcalase). Protein solutions were pre-incubated at the reaction temperature for 10 min prior to the addition of either 172 U/mg protein of porcine pepsin (EC 3.4.23.1, 3440 U/mg, Sigma-Aldrich), 0.025 U/mg of Neutrase or 0.005 U/mg of alcalase (both from Novozymes A/S, Bagsvaerd, Denmark). Hydrolyses were conducted, under continuous shaking, at 37ºC for 24 h with pepsin or at 50ºC for 60 min with Neutrase and alcalase. The reaction with pepsin was neutralized to pH 7.0 and all the enzymes were inactivated by heating at 95ºC for 15 min. The samples were then centrifuged at 5000 x g for 10 min (4ºC), the supernatants collected and, when required, fractionated using high performance ultrafiltration units of 10 and 3 kDa cut-off (Amicon Ultra, Millipore, Eschborn, Germany). The hydrolysates and their fractions were stored at -20ºC prior to subsequent analyses. The protein content of the hydrolysates and their fractions was determined using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific) and the resulting values were used to standardize their concentration for *in vitro and ex vivo* experiments. For *in vivo* assays, the protein content of the hydrolysates was determined by the Kjeldahl method.

3.2. Analytical techniques

3.2.1. Characterization of the hydrolysates by RP-HPLC

The hydrolysates and their fractions were analysed in a Waters 600 HPLC system (Waters, Milford, MA, USA) using a RP318 column (250x4.6mm, Bio-Rad, CA, USA). The operating conditions were: flow rate, 1 mL/min; injection volume, 50 μL; solvent A, 0.37 mL/L trifluoroacetic acid (TFA) in Milli-Q water; and solvent B, 0.27 mL/L TFA in HPLC grade acetonitrile (ACN). Elution was performed with a linear gradient of solvent B in A going from 0 to 70% in 75 min, followed by 100% B for 30 min. Absorbance was recorded at 214 nm with a Waters 2487 dual detector and the software Empower 2000 system data (Waters) was used.

3.2.2. Isolation of peptide fractions by chromatographic techniques

Semi-preparative RP-HPLC was performed on a Waters Series 600 HPLC equipped with a Millennium 3.2 Software for data acquisition (Waters), using a Prep Nova Pak® HR C18 column (300 x7.8 mm i.d., 6 μm particle size; Waters). The operating conditions were: column temperature, 30ºC; flow rate, 4 mL/min; injection volume, 400 μL (4 mg of protein); solvent A, 1 mL/L TFA in Milli-Q water and solvent B, 0.8 mL/L TFA in HPLC grade ACN. Elution was conducted with a linear gradient of solvent B in A from 0 to 60% B in 100 min, followed by washing with 100% B and conditioning the column. Five fractions were collected using a Waters Fraction Collector II. ACN was removed by rotary evaporation and aliquots were lyophilized for further use.

Alternatively, fractionation was also conducted by anion exchange chromatography on an ÄKTA Explorer FPLC System (GE Healthcare, Freiburg, Germany) using a Mono Q HR 5/5 column (50 x 5 mm i.d., 10 μm particle size; GE Healthcare). The operating conditions were: flow rate, 1 mL/min; injection volume 1 mL (10 mg of protein); solvent A, 20 mM Tris pH 9.0 and solvent B, 20 mM Tris pH 9.0 containing 1M NaCl. Elution was conducted with a linear gradient of solvent B in A from 0 to 100% B in 15 min, followed by washing with 100% B and

conditioning the column. Two fractions were collected using a Fraction Collector Frac-950 (GE Healthcare). These were purified by solid phase extraction on OASIS HLB Plus-short-cartridges (225 mg, 60 μm; Waters) and lyophilized for further use.

3.2.3. Peptide mass distribution by MALDI-TOF

Peptide mass distribution of the hydrolysates was analyzed by MALDI-TOF using a Bruker AUTOflex Speed spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The hydrolysates (0.5 μL dissolved at a concentration of 5 μg/mL) were loaded on a dry 2.5-dihydroxybenzoic acid (DHB) matrix spot (0.5 μL of 20 mg/mL DHB in ACN/methanol, 70/30%, containing 1% TFA) onto a Bruker Anchorchip target. All mass spectra were initially calibrated with Peptide Calibration Standard and Protein Calibration Standard I (Bruker Daltonik). Mass spectra were acquired in positive reflection, by summing 50 laser pulses at a fixed slide target position, using a 337 nm nitrogen laser and an acceleration voltage of 20 kV. Molecular mass detection limit was 562 Da due to signals arising from fragments and adduct ions of the DHB matrix.

3.2.4. Peptide identification by RP-HPLC-MS/MS

RP-HPLC with UV detection (214 nm), connected on-line to an electrospray ion source and a quadrupole ion trap mass analyser (ESI-MS/MS), was performed using an Agilent 1100 Series HPLC equipment (Agilent Technologies, Waldbronn, Germany) with a Hi-Pore® Reversed Phase RP-318 Column (250 x 4.6 mm i.d.; Bio-Rad) and an Esquire 3000 mass spectrometer (Bruker Daltonik). To aid the identification of disulphide linked fragments, the samples were analyzed after a reducing step using dithiothreitol (DTT), at a final concentration of 70 mM and pH 7.0, for 1 h at 37ºC (Chicón et al., 2006).

The operating conditions were: flow rate, 0.8 mL/min; injection volume, 50 μL; solvent A, 0.37 mL/L TFA in Milli-Q water and solvent B, 0.27 mL/L TFA in HPLC grade ACN. Elution was conducted with a linear gradient of solvent B in A from 0 to 70% in 75 min, followed by 100% B for 30 min. Ion source parameters were: nebulizer pressure, 60 psi; dry

gas, 8 L/min and dry temperature, 350ºC. Using Data Analyses TM (version 3.0; Bruker Daltonik), the m/z spectral data were processed and transformed to spectra representing mass values. Biotools (version 2.1; Bruker Daltonik) was used to process the MS(n) spectra and Mascot software (version 2.3.3, Matrix Science, London, UK) to perform peptide sequencing. For each sample, a minimum Mascot score corresponding to $P < 0.05$ was considered as a pre-requisite for validation of peptide identification.

3.3. Cell cultures

3.3.1. Murine spleen and mesenteric lymph node cells stimulated with mitogens

Nine 6-week-old female BALB/c mice, obtained from Charles River Laboratories (Saint Germain sur l´Arbresle, France), were sacrificed by CO_2 inhalation. Whole spleens and MLNs were excised, separately pooled in 3 groups (which were used to perform biological replicates) and processed as described by Jiménez-Saiz et al. (2011). Splenocytes and MLN cells were cultured in RPMI 1640 supplemented with 10% of fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 μg/mL) (all from Biowest SAS, Nuaillé, France), at a cellular density of $4x10^6$ cells/mL in 48-well plates (non-stimulated control), and incubated with either 1 μg/mL ConA or 1 μg/mL LPS (both from Sigma-Aldrich) and different concentrations (20-200 μg/mL) of the egg white proteins and their hydrolysates (Sharma et al., 2007). The effect of the heat-inactivated enzymes was evaluated in a separate experiment, using splenocytes isolated from 4 mice pooled in 2 groups and incubated with a concentration of enzyme equivalent to that present in 200 μg/mL of the hydrolysates. The supernatants were collected after 72 h of culture in a 5% CO_2 incubator at 37ºC, and stored at -80ºC until their analyses.

3.3.2. Th2-primed human peripheral blood mononuclear cells

Th2-skewed PBMCs were obtained as previously described by Holvoet et al. (2013). Human blood from 8 healthy donors was sterilely collected into EDTA tubes. PBMCs were isolated by density gradient centrifugation on Ficoll-Histopaque (GE healthcare) and

resuspended in IMDM medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin and 0.1% gentamicin (all from Biowest SAS) at a cellular density of $1.5x10^6$ cells/mL in 48-well plates. PBMCs were stimulated with 50 U/mL of IL-4 (Peprotech, Hamburg, Germany) and 1µg/mL of anti-CD40 antibody (eBioscience, San Diego, CA, USA) to induce a Th2 phenotype. After 3 days in a 5% CO_2 incubator at 37ºC, the intact proteins and their hydrolysates were added at different concentrations (20-200 µg/mL) and cultured for a further 3 days in triplicate. As positive control, 1µg/mL of LPS (Sigma-Aldrich) was used. Inactivated enzymes were also tested at a concentration equivalent to that present in 200 µg/mL of the hydrolysates. Following incubation, the supernatants were collected and stored at -80ºC until their analysis.

3.3.3. Th1-primed human peripheral blood leukocytes

PBLs were isolated from human blood from 10 healthy donors and Th1-skewed as described by Richard et al. (2005). Blood was mixed with one volume of 3% (w/v) dextran (Sigma-Aldrich) in 0.9% (w/v) NaCl solution. After 30 min of incubation, PBLs were recovered from the upper layer, erythrocytes were lysed in 0.2% (w/v) NaCl for 30 s, and isotonic osmolarity was re-established by adding one volume of 1.6% (w/v) NaCl. Isolated PBLs were cultured in RPMI 1640 medium supplemented with 0.25% of FBS, 1% L-glutamine and 0.5% penicillin/streptomycin (all from Biowest SAS) at a cellular density of $1x10^6$ cells/mL in 48-well plates. PBLs were stimulated with 100 ng/mL of LPS (Sigma-Aldrich) and 20 U/mL of IFN-γ (Peprotech) for 24 h. The intact proteins, their hydrolysates and low molecular mass fractions (< 10 and < 3 kDa), were added together with LPS/IFN-γ at a concentration of 200 µg/mL in triplicate. Inactivated enzymes were also tested at a concentration equivalent to that present in 200 µg/mL of the hydrolysates. The supernatants were collected and stored at -80ºC until their analysis.

3.3.4. Spleen cells from mice sensitized to egg white proteins

Six-week-old female specific-pathogen-free BALB/c mice, purchased from Charles River Laboratories, were sensitized with 1 mg of either OVA, OM or LYS, poly-sensitized with a mixture of 1 mg of each of the three proteins or sensitized with 5 mg of EW, using CT as adjuvant, as described in section 3.4.1. Naïve mice just received sterile phosphate buffered saline (PBS). Each group consisted of 5 mice. On day 49, mice were euthanized by CO_2 inhalation and spleen cells were collected and processed under sterile conditions. Pooled splenocytes from each group (n=5) were cultured in 48-well plates (4×10^6 cells/mL) and stimulated in triplicate with ConA (2.5 μg/mL), as positive control; RPMI-1640 medium, as negative control; and the intact proteins, their hydrolysates or peptide fractions at different concentrations (10-200 μg/mL). Inactivated enzymes were also tested at a concentration equivalent to that present in 200 μg/mL of the hydrolysates. The immunomodulating effect of the hydrolysates and their selected fractions, at a concentration of 200 μg/mL, was also tested concomitantly with the intact allergens (200 μg/mL). Supernatants were collected after 72 h at 37°C in 5% CO_2, and stored at -80°C until their analysis.

3.3.5. Cell proliferation and viability

The proliferation assays were carried out as described by Quah and Parish (2012), with some modifications. Before their culture, cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) (5 μM in non-supplemented RPMI culture medium) using a CellTrace CFSE cell proliferation kit (Life Technologies, Carlsbad, USA) and incubated at 37ºC for 10 min. The reaction was stopped by adding 5 volumes of supplemented RPMI culture medium and cells were then incubated at 4ºC for 10 min, followed by 3 washes with PBS. After incubation period with the stimuli, cells were harvested and analytical flow cytometry was performed using a Gallios instrument (Beckman Coulter, Krefeld, Germany). Statistical analysis of CFSE distributions was based on histogram profiling using Overton subtraction as applied by

the FlowJo software version 9.1 (Treestar Inc., Ashland, OR, USA). The results were expressed as the percentage of cells in the final population that divided at least once.

Cytotoxicity of the hydrolysates was determined on spleen cell cultures using the 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazoliumbromide (MTT) method described by Hansen et al. (1989), with modifications. After incubation with the stimuli in 96-well plates, 150 µL of 1 mg/mL MTT (Sigma-Aldrich) were added to each well and incubated for a further 3 h. The plates were centrifuged at 500 x g for 10 min and the supernatants discarded. The formazan precipitate was dissolved by the addition of 50 µL of dimethyl sulfoxide and the absorbance of each well was measured at 570 nm in a plate reader (Multiskan FC, Thermo Scientific). Cell viability was expressed as percentage of the value of the corresponding control depending of the stimulus.

3.3.6. Intracellular reactive oxygen species

The intracellular formation of ROS was assessed using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. After the incubation period, cells were labelled with 2.5 µM DCFH-DA in Hanks balanced salt solution (Biowest SAS) and incubated in dark for 45 min at 37ºC (Cosentino et al., 2008). Afterwards, cells were washed twice with PBS and subjected to chemical oxidative stress with 0.5 mM *tert*-butyl hydroperoxide (*t*-BOOH) (Sigma-Aldrich) for 15 min. Fluorescence emission due to the oxidation of DCFH-DA by intracellular ROS was read at excitation and emission wavelengths of 485 nm and 528 nm, respectively, in a Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany).

3.4. *In vivo* experiments

Three- to six-week-old female specific-pathogen-free BALB/c mice were purchased from Charles River Laboratories, housed under standard conditions and fed an animal protein-free diet (SAFE, Route de Saint Bris, France).

3.4.1. Sensitization and challenge protocols

In the sensitization experiments, six-week-old mice (n=5 per group) were administered by intragastric gavage, during three consecutive days on the first week and once per week during the following 5 weeks, the antigen (1 mg of either OVA, OM or LYS, a mixture of 1 mg of each of the three proteins, 5 mg of EW or 5 mg of either OA or OP) plus 10 μg of CT (List Biologicals, Campbell, USA). Sham-sensitized mice only received 10 μg of CT.

Challenges were performed one week after the last sensitization dose. Oral challenge with 50 mg of antigen per mouse was followed by i.p. challenge with 100 μg of antigen per mouse, 40 min apart. 30 min after each challenge (oral and i.p.), anaphylactic responses were evaluated by scoring clinical signs and rectal temperature as described in Pablos-Tanarro et al. (2016). Blood and faecal samples were collected at different time points throughout the experiments. Three hours after the last challenge, mice were euthanized by CO_2 inhalation, and samples from duodenum, MLNs and PPs collected for further analyses. Spleens were individually excised and isolated cells were cultured with RPMI-1640 medium (as negative control) or 200 μg/mL of the egg white proteins or their hydrolysates as described in section 3.3.4.

3.4.2. Preventive and therapeutic treatments

The preventive potential of OVA and its hydrolysates was evaluated using 5 groups of three-week-old mice (n=5) which were orally pre-treated with PBS (two groups), OVA, OA or OP (5 mg per mouse) 3 times per week during 3 weeks. One week after the last preventive dose, a group treated with PBS and the groups treated with OVA, OA and OP were sensitized to EW as indicated, while the remaining group treated with PBS only received 10 μg of CT during the sensitization period. All groups were orally and i.p. challenged with EW, as described above.

Finally, the therapeutic effect of the intact protein and its hydrolysates was studied in 5 groups of six-week-old mice (n=5), four of them sensitized to EW and one sham-sensitized. Three groups were orally treated with OVA, OA or OP (5 mg per mouse) 3 times per week

during 3 weeks. An additional group sensitized to EW and the sham-sensitized group just received PBS during the treatment period. Three days after the completion of the treatment period, all the groups were orally and i.p. challenged with EW, as described above. Mice treated with OVA and OP were kept for a further three weeks without any treatment, in order to evaluate the maintenance of the tolerance status acquired and challenged 3 days after. Blood, faeces and intestinal tissue samples were collected as indicated in section 3.4.1.

3.4.3. Passive cutaneous anaphylaxis assays

PCA tests were performed as described by López-Expósito et al. (2012). Briefly, naïve BALB/c mice were injected intradermically in the right ear pinna with 20 µL of pooled sera (n=5) from OVA-, LYS- or OM-sensitized mice and with pooled sera from naïve BALB/c mice in the left ear pinna. Twenty four hours later, mice (2 per test) were injected intravenously with 200 µg of the respective intact antigen (OVA, OM or LYS) or the corresponding hydrolysates in 100 µL of 1% Evans blue dye (Sigma-Aldrich). One hour after protein administration, mice were euthanized by CO_2 inhalation and ears were individually collected and incubated overnight in N,N-dimethylformamide (Sigma-Aldrich). The absorbance of the supernatant was measured at 655 nm in order to quantify the extravasation of the dye.

The participation of IgE and IgG1 in the induction of anaphylaxis in the BALB/c model was evaluated following the protocol described above, with some modifications. Shortly, naïve mice (2 per test) were injected with 20 µL of either unheated or heated (56°C for 3 h to inactivate IgE; Li et al., 2000) pooled sera from sensitized mice, and with pooled sera from sham-sensitized mice in the left ear pinna (all serum samples were collected after sacrifice). Twenty four hours later, mice were injected intravenously with 200 µg of the respective antigen in 100 µL of 1% Evans blue dye (Sigma-Aldrich).

3.5. Quantification of cytokines, antibodies and mast cell degranulation

3.5.1. Cytokines in cell culture supernatants

The levels of IL-4, IL-5, IL-13, IL-8, IL-10, IL-17, IL-12p70, TNF-α and IFN-γ in the supernatants of cell cultures after incubation with the stimuli were quantified by ELISA or Luminex commercial kits according to the manufacturer's instructions (eBioscience).

3.5.2. Mouse IgG isotypes in cell culture supernatants

The levels of total IgG1 and IgG2a in the supernatants of cells from section 3.3.1 were determined by ELISA. Briefly, 96-well plates were coated with 0.1 µg/well of anti-mouse IgG1 and IgG2a (BD Biosciences, San José, CA, USA) and, after an overnight incubation at 4ºC, plates were blocked and incubated at 4ºC with diluted culture supernatants (1:5 v/v) for 12 h. Plates were then incubated with 0.2 µg/well of biotinylated anti-mouse IgG1 and IgG2a, followed with avidin-horseradish peroxidase (HRP) (all from BD Biosciences). A signal amplification system, based on the subsequent addition of tyramide-biotin and streptavidin-HRP, was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Waltham, MA, USA). The reactions were developed using ABTS as substrate (Roche, Mannheim, Germany) and read at 405 nm in a plate reader (Multiskan FC, Thermo Scientific).

3.5.3. Human IgE in cell culture supernatants

The levels of IgE in the supernatants of cells from section 3.3.2 were determined by ELISA. 96-well plates were coated with 0.2 µg/well of anti-human IgE (Dako, Glostrup, Denmark) and, after an overnight incubation at 4ºC, plates were blocked and incubated at 4ºC with diluted culture supernatants (1:2 v/v) for 12 h. Detection was carried out by adding 0.5 µg/well of biotinylated anti-human IgE (Dako), followed with an avidin-HRP incubation step (BD Biosciences). The reactions were developed using ABTS as substrate (Roche) and read at 405 nm.

3.5.4. Serum and faecal levels of antigen-specific antibodies

Mouse sera were obtained by centrifugation at 2500 x g for 10 min and kept at -20ºC until analysis, and faeces were lyophilised. EW-specific IgE, IgG1 and IgG2a and those against the hydrolysates (OA and OP) were quantified by ELISA as previously explained by Pablos-Tanarro et al. (2016) with some modifications. Briefly, 96-well plates were coated with 0.5 µg/well of either EW, OA, OP or with 0.1 µg/well of anti-mouse IgE, IgG1 and IgG2a (BD Biosciences) for the reference curves. After an overnight incubation at 4ºC, plates were blocked and incubated with diluted (v/v) serum samples (1:20 for IgE, 1:2000 for IgG1 and 1:100 for IgG2a against EW; 1:5 for IgE, 1:200 for IgG1 and 1:50 for IgG2a against OA and OP) or serial dilutions of mouse IgE, IgG1 and IgG2a isotypes (BD Biosciences). Detection was carried out by adding 0.2 µg/well of biotinylated anti-mouse IgE, IgG1 and IgG2a, followed with an avidin-HRP incubation step (BD Biosciences). Reactions were developed with ABTS as substrate (Roche) and read at 405 nm. Detection of specific IgA against EW, OA and OP was carried out in the supernatants of lyophilized faecal samples homogenized in PBS during 3 h and centrifuged at 4000 x g for 10 min. Quantification of IgA levels was performed as described above except for the use of anti-mouse IgA antibodies and IgA isotype control for the reference curves (BD Biosciences). Faecal supernatants were diluted 1:50 (v/v) for EW-specific IgA and 1:5 (v/v) for IgA specific against the hydrolysates.

3.5.5. Human IgE-binding by inhibition ELISA

Human IgE binding of the samples was assessed by inhibition ELISA as previously reported (Jiménez-Saiz et al., 2013), using a pool of 8 different sera from egg allergic children with IgE specific to OVA, LYS and OM ranging between 35.2-1326.5 kU/L, 2.3-36.6 kU/L and 19.3-120.0 kU/L, respectively. Briefly, OVA, LYS and OM were used as coating antigens (0.5, 2.5 and 1.75 µg, respectively). Serial dilutions of the hydrolysates were incubated with the pooled sera (1:1 v/v) and, after 2 h, added to the appropriate coated plate. Detection was carried out by adding 0.1 µg/well of polyclonal rabbit anti-human IgE (Dako), followed with 0.2

µg/well of polyclonal swine anti-rabbit immunoglobulin HRP-labelled (Dako). A tyramide-biotin and streptavidin-HRP amplification system was employed, following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences). The reactions were developed using TMB as substrate, stopped with 0.5 M sulfuric acid and the absorbance read at 450 nm in a plate reader (Multiskan FC, Thermo Scientific). Results were expressed as EC_{50} value, that is, the effective sample concentration (µg/mL) for 50% of the maximum binding to IgE.

3.5.6. Mouse IgG1-binding by inhibition ELISA

Mouse IgG1-binding capacity was assessed by inhibition ELISA, coating the plates with 0.5 µg/well of EW or OA. Serial dilutions of samples (EW, OP, OA and its fractions below and above 10 kDa, as well as the alcalase enzymatic extract) were incubated with pooled sera (1:1 v/v) from mice administered EW or OA plus CT and, after 2 h of incubation, added to the appropriate coated plate. Detection was carried out by adding 0.2 µg/well of biotinylated anti-mouse IgG1, followed with an avidin-HRP incubation step (BD Biosciences). The reactions were developed using TMB (Sigma-Aldrich) as substrate, stopped with 0.5 M sulfuric acid and the absorbance read at 450 nm. Results were expressed as B/Bo, where B and Bo represent, respectively, the amount of IgG1 bound to immobilised antigen (EW or OA) in the presence and absence of a known concentration of inhibitors (EW, hydrolysates, OA fractions or the enzyme).

3.5.7. Serum levels of mouse mast cell protease I

Mast cell degranulation was evaluated in serum samples collected after oral and i.p. challenges by determining the concentration of mMCP-1 using a commercial ELISA kit according to the manufacturer's instructions (eBioscience).

3.6. Relative gene expression

Total RNA was individually extracted from duodenum tissues and cells isolated from MLNs and PPs by using NucleoSpin RNA Kit (Macherey-Nagel Gmbh & Co., Düren,

Germany), according to the manufacturer's instructions. Duodenum samples were previously homogenized using an Ultra-Turrax T8 (IKA Labortechnik, Staufen, Germany). Total RNA was qualitatively evaluated and quantified with an Agilent 2100 Bioanalyzer (Agilent Technologies), and 2 µg were used to synthesize cDNA using PrimeScript RT reagent kit (TaKaRa Bio Inc., Shiga, Japan).

qPCR was performed in a real-time thermocycler (ViiA7 Real-Time PCR System, Applied Biosystems, Foster, USA) using SYBR Premix ExTaq II (Tli RNaseH Plus) (TaKaRa). The primer pairs, shown in table 3.1, were purchased from Integrated DNA Technologies (IDT, Coralville, USA) and used at a final reaction concentration of 166.7 nM (for β-actin, IL-10 and TGF-β) or 312.5 nM (for IL-33, IL-25, IL-17, TSLP, GATA-3, T-bet and Foxp3). The thermal cycling conditions included 2 min incubation at 50°C, followed by 10 min pre-incubation at 95°C, and 40 cycles consisting on a denaturation step of 15 s at 95°C and, for primer annealing and polymerase extension, 45 s at 58ºC followed by 15 s at 60ºC (for β-actin, IL-10 and TGF-β) or 60 s at 60°C (for IL-33, IL-25, IL-17, TSLP, GATA-3, T-bet and Foxp3). Melting curve analysis was used to confirm specific product formation. The efficiency of each qPCR reaction was estimated between 90% and 110%. The relative gene expression was calculated by normalizing data to the expression of the reference gene β-actin, using the corresponding sham-sensitized group of each experiment (which only received CT and was treated, if applicable, with PBS) as calibrator, as described by Livak and Schmittgen (2001). All the amplification reactions were performed in triplicate.

3.7. T cell subsets by flow cytometry

Isolated spleen cells were recovered in a solution of PBS containing 2% FBS and 1mM EDTA and, afterwards, Fc receptors were blocked using anti-CD16/CD32 (clone 2.4G2, BD Biosciences). The determination of live cells for analyses was conducted using LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Scientific). T cell subsets were analysed using the follow antibodies: anti-CD4-Alexa Fluor 700 (clone GK1.5), anti-CD69-PerCP-Cy5.5 (clone

H1.2F3), anti-CXCR3-APC (clone CXCR3-173), anti-ST2-PE (clone RMST2-2), anti-CD25-FITC (clone 3C7) and anti-CD39-PE-Cy7 (clone Duha59; all from Biolegend, San Diego, US). Approximately 1 x 10^5 cells were acquired using a Gallios flow cytometer (Beckman Coulter). All the analyses were performed using Kaluza Analysis software (version 1.3, Beckman Coulter). Samples were first inspected in all light scatter patterns and fluorescence channels to confirm quality and abnormal population were excluded. Unlabelled processed samples were used for reporting the percentage of positive cells.

Table 3.1. Primer sequences used for RT-qPCR analyses of relative gene expression.

Gen	Primer pair	Reference
β-actin	*fw* 5´ AGCTGCGTTTTACACCCTTT 3´ *rv* 5´ AAGCCATGCCAATGTTGTCT 3´	Cardoso et al., 2009
FoxP 3	*fw* 5' ACAACCTGAGCCTGCACAAGT 3' *rv* 5' GCCCACCTTTTCTTGGTTTTG 3'	Cardoso et al., 2009
GATA 3	*fw* 5' CCTTAAAACTCTTGGCGTCC 3' *rv* 5' AGACACATGTCATCCCTGAG 3'	Zhang et al., 2013
IL-10	*fw* 5' GCCTTATCGGAAATGATCCA 3' *rv* 5' AGGGGAGAAATCGATGACAG 3'	Yang et al., 2009b
IL-17	*fw* 5' TGCCTGTGGCACTGAAGTAG 3' *rv* 5' TTCATGGCTGCAGTGAAAAG 3'	Lenoir et al., 2016
IL-25	*fw* 5' ACAGGGACTTGAATCGGGTC 3' *rv* 5' TGGTAAAGTGGGACGGAGTTG 3'	Li et al., 2013
IL-33	*fw* 5' ATTTCCCCGGCAAAGTTCAG 3' *rv* 5' AACGGAGTCTCATGCAGTAGA 3'	Li et al., 2013
T-bet	*fw* 5' GTATCCTGTTCCCAGCCGTTTC 3' *rv* 5' ACTGTGTTCCCGAGGTGTCC 3'	Zhao et al., 2010
TGF-β	*fw* 5' TTGCTTCAGCTCCACAGAGA 3' *rv* 5' TACTGTGTGTCCAGGCTCCA 3'	Yang et al., 2009b
TSLP	*fw* 5' AGGCTACCCTGAAACTGAGA 3' *rv* 5' GGAGATTGCATGAAGGAATAC 3'	Negishi et al., 2012

fw, forward primer; *rv*, reverse primer

3.8. Ethics statement

All protocols involving animals followed the European legislation (Directive 2010/ 63/UE) and were approved by the CSIC Bioethics Committee and the Comunidad de Madrid (Ref PROEX 089/15). All human samples were obtained with written consent from the donors involved in the study and all experiments were approved by the CSIC Bioethics Committee.

3.9. Statistical analyses

Data were subjected to one-way ANOVA followed by *post hoc* multiple-comparison using the Tukey's test, except for results derived from the assays with human peripheral blood cells (section 4.2) where ANOVA analyses were followed by Dunnett's *post hoc* multiple-comparison test. Statistical differences of the clinical sign scores and gene expression results were evaluated by Mann-Whitney U test. In all cases, *P* values< 0.05 were considered to be statistically significant. The statistical analyses were performed using GraphPad Prism v5 (GraphPad Software Inc., San Diego, USA) and STATISTICA software (Version 7.1; StatSoft Inc., 2006, www.statsoft.com).

4. RESULTS AND DISCUSSION

4.1. Hydrolysates of egg white proteins modulate T and B cell responses in mitogen-stimulated murine cells

During the past few years, increasing evidence has pointed out the existence of peptides which specifically act on the immune system. The immunomodulating activity of these peptides comprises effects on cell migration, survival and proliferation, as well as stimulation of antimicrobial and immune signalling molecules (Easton et al., 2009). Although existing studies on the effects of immunomodulating peptides have focused mainly on cells of the innate immune system, there is evidence that they alter T and B lymphocyte functions and could contribute to a balanced Th1 and Th2 response (Hilchie et al., 2013).

Whereas the use of known bioactive peptides as templates to synthetize optimized peptides with the ability to modulate the immune response has proved effective in the formulation of new sequences with better properties, the high cost associated with generating synthetic peptides on a large scale is an important obstacle to their production (Easton et al., 2009). In this respect, hydrolysis of food proteins appears as an attractive and safe alternative for a reproducible and standardized production of immune-active peptides with low costs that would allow their consumption in the form of functional foods. However, while a number of potentially immunomodulating peptides have been described as arising from food proteins, such as milk whey proteins, contradictory results are frequently reported regarding their biological properties, which could be due to the use of assay systems that fail to correlate with a specific immune response or to the contamination of the peptide preparations with immunostimulating substances (Brix et al., 2003; Gauthier et al., 2006).

Egg white protein hydrolysates are good candidates for non-pharmacological approaches to prevent and treat complex diseases by virtue of their multifunctional properties, which comprise antihypertensive, antioxidant, antimicrobial and lipid-lowering activities (García-Redondo et al., 2010; Yu et al., 2014). As a first step in the search for immunomodulating enzymatic hydrolysates of egg proteins, we used as an assay method an *in vitro* model system of murine cells stimulated with T and B cell mitogens (Fig. 4.1.1). Hydrolysates of OVA, LYS, OM and EW with pepsin, Neutrase and alcalase were produced and their constituent peptides characterized by RP-HPLC and MALDI-TOF. Subsequently, the

effects of these hydrolysates on cytokine secretion, antibody production, oxidative stress and proliferation of murine spleen and MLN cells stimulated with ConA and LPS were assessed (Fig. 4.1.1).

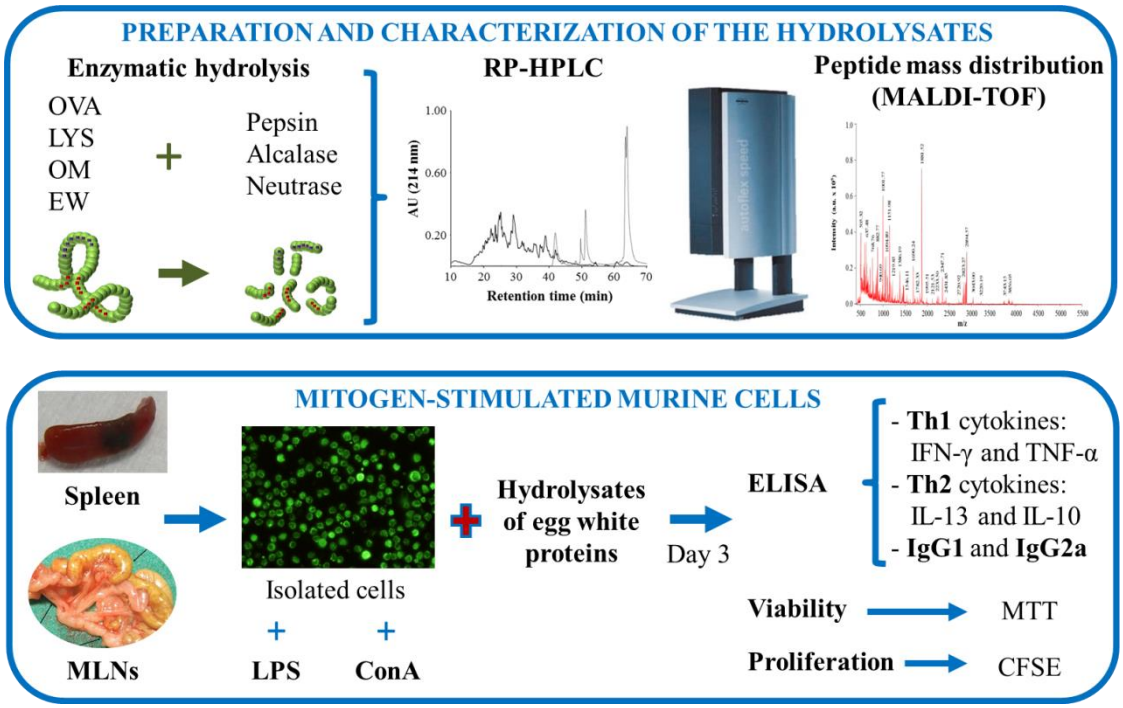

Figure 4.1.1. Scheme of the experimental procedure followed in section 4.1. CFSE, method based on carboxyfluorescein succinimidyl ester; ConA, concanavalin A; EW, whole egg white; LPS, lipopolysaccharide; LYS, lysozyme; MLNs, mesenteric lymph nodes; MTT, method based on 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazoliumbromide; OM, ovomucoid; OVA, ovalbumin; RP-HPLC, reverse phase high-performance liquid chromatography.

4.1.1. Results

4.1.1.1. Egg white protein hydrolysates with alcalase are characterized by low molecular mass peptides

Individual egg white proteins (OVA, LYS and OM) and EW were hydrolyzed with pepsin, Neutrase and alcalase. The hydrolysis conditions were optimized to obtain a complete degradation of the substrates and the absence of intact proteins was confirmed by RP-HPLC (Fig. 4.1.2). As expected, OVA was quite resistant to pepsin action (Benedé et al., 2014), and its hydrolysis required 24 h at 37ºC. LYS and OM also disappeared after 24 h of hydrolysis with pepsin at 37ºC and pH 1.5. OM is usually readily digested by pepsin, but it gives rise to

proteolysis fragments linked by disulfide linkages, which is also the case of pepsin-treated LYS, because these proteins are stabilized by 9 and 4 disulfide bridges, respectively (Benedé et al., 2013; Jiménez-Saiz et al., 2014). Neutrase and alcalase hydrolyzed the egg white proteins in less than 1 h at 50ºC, yielding similar chromatographic profiles.

The peptide mass distribution of the hydrolysates was analysed by MALDI-TOF (Table 4.1.1). The occurrence of unspecific signals from DHB matrix, such as small fragments and adduct ions, established the limit of detection in 562 Da. Molecular masses lower than 10 kDa were found in all cases, although, comparatively, alcalase led to the smallest fragments: 85-97% of the peptides were lower than 1500 Da, with more than 50% of them between 601 and 900 Da. Almost 30% of the peptides present in the hydrolysates produced with pepsin and Neutrase had molecular masses higher than 1500 Da.

Table 4.1.1. Peptide mass distribution in the hydrolysates of OVA (O), LYS (L), OM (M) and EW (E) with pepsin (P), Neutrase (N) and alcalase (A). The masses included in each mass range are expressed as percentage of all the masses detected by MALDI-TOF analyses.

Mass Range (Da)	**Frequency (%)**											
	OP	**ON**	**OA**	**LP**	**LN**	**LA**	**MP**	**MN**	**MA**	**EP**	**EN**	**EA**
≤ 600	7.72	6.97	15.52	5.29	4.79	13.33	3.41	4.23	8.17	4.86	3.42	5.96
601-900	37.40	30.31	55.75	32.60	35.21	50.67	30.30	23.78	49.03	35.07	28.57	50.47
901-1200	24.39	20.91	22.99	17.18	22.25	19.11	17.80	14.98	21.40	25.00	19.88	19.44
1201-1500	6.50	11.50	2.87	14.54	18.03	10.67	9.85	12.05	12.06	15.63	11.49	9.72
1501-3000	10.98	21.25	0.57	25.99	14.37	4.00	18.18	15.31	7.00	6.25	22.36	6.90
≥ 3001	13.01	9.06	2.30	4.41	5.35	2.22	20.45	29.64	2.33	13.19	14.29	7.52

4.1.1.2. Egg white protein hydrolysates modulate T cell responses in ConA-stimulated splenocytes

Incubation of splenocytes from BALB/c mice with ConA, a mitogen able to stimulate mouse T lymphocytes (Sharma et al., 2007), induced a vigorous T cell proliferation, as assessed by CFSE labelling (Table 4.1.2), and secretion of Th1 (IFN-γ and TNF-α, Fig. 4.1.3a and b) and

Th2 cytokines (IL-13 and IL-10, Fig. 4.1.3c and d). The concomitant addition of the hydrolysates of OVA, LYS and OM with pepsin, Neutrase and alcalase did not significantly affect the release of IFN-γ by ConA-stimulated cells (Fig. 4.1.3a). However, the hydrolysates modified the production of TNF-α in a dose-dependent manner, with the lowest dose (20 μg/mL) usually reducing the ConA-induced release of TNF-α, which subsequently increased with the hydrolysate concentration (particularly in the hydrolysate of OVA with pepsin, as well as the hydrolysates of OVA, LYS and OM with Neutrase). A low TNF-α production was detected as a result of the addition of the hydrolysate of LYS with pepsin, although intact LYS and OM also decreased the release of TNF-α at the three concentrations tested (Fig. 4.1.3b). Regarding Th2 cytokines, the ConA-induced secretion of IL-13 decreased with increasing concentrations of the hydrolysates of OVA, LYS and OM with alcalase and LYS with pepsin, while the highest concentration of the hydrolysates of OVA and OM with pepsin (200 μg/mL) led to an increase in the release of IL-13 (Fig. 4.1.3c). IL-10 secretion by ConA-stimulated splenocytes decreased following incubation with the hydrolysates of OVA with alcalase and LYS with pepsin, as well as with intact LYS and OM at the highest concentrations (Fig. 4.1.3d).

The effects exerted by hydrolysates of EW were then assessed. As shown in Fig. 4.1.3a, the levels of IFN-γ were not significantly influenced by the addition of the hydrolysates. However, the highest concentrations of the hydrolysate of EW with Neutrase increased the ConA-induced production of TNF-α, IL-13 and IL-10, while the hydrolysate with alcalase reduced the production of TNF-α, IL-13 and IL-10 (Fig. 4.1.3b-d).

Altogether, these results indicate that, while intact OVA increased TNF-α, intact LYS and OM reduced the levels of TNF-α, IL-13 and IL-10 and EW, those of IL-13 and IL-10; the hydrolysates produced by Neutrase enhanced Th1 responses (ON, LN, MN and EN; Fig. 4.1.3b) and, to a lesser extent, Th2 responses (EN; Fig. 4.1.3c) and those produced by alcalase reduced Th2 responses (OA, LA, MA and EA; Fig. 4.1.3c). On the other hand, the hydrolysate of LYS with pepsin exerted a suppressive effect on both Th1 (TNF-α) and Th2 mediators (IL-13 and

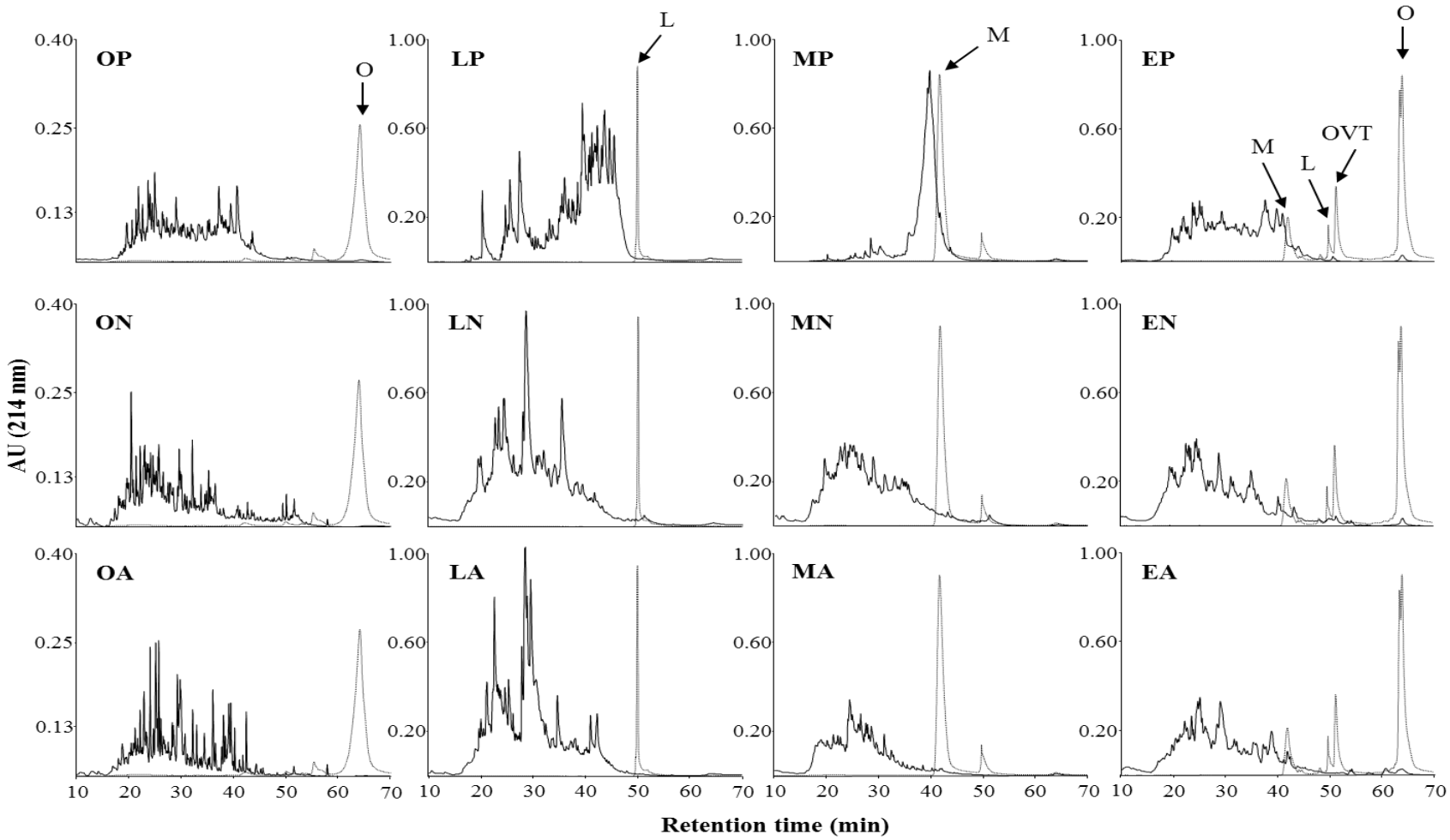

Figure 4.1.2. RP-HPLC patterns of OVA (O), LYS (L), OM (M) and EW (E) hydrolysates produced with pepsin (P), alcalase (A) and Neutrase (N). OVT designates ovotransferrin. The profile of the respective intact protein is drawn with a pale grey line.

Table 4.1.2. Effects of OVA (O), LYS (L), OM (M), EW (E) and their hydrolysates with pepsin (P), Neutrase (N) and alcalase (A) on proliferation and viability of splenocytes. Proliferation of ConA- and LPS-stimulated splenocytes cultured with 200 μg/mL of the intact proteins and their hydrolysates was assessed by CFSE labelling and the results expressed as the percentage of cells in the final population that divided at least once. Viability was measured by the MTT assay either on non-stimulated (RPMI), ConA- or LPS- stimulated splenocytes (taken as 100%) and the corresponding percentage was calculated in the cultures treated with 200 μg/mL of the proteins and their hydrolysates.

	Proliferation ConA-stimulated cells	**Proliferation LPS-stimulated cells**	**Viability RPMI**	**Viability ConA-stimulated cells**	**Viability LPS-stimulated cells**
RPMI	4.27 ± 0.10 *	12.95 ± 0.78 *	100.00 ± 0.00	-	-
ConA	74.25 ± 0.49	-	-	100.00 ± 0.00	-
LPS	-	29.30 ± 0.28	-	-	100.00 ± 0.00
O	75.95 ± 0.92	25.60 ± 0.99	152.12 ± 16.35	106.30 ± 1.35	79.91 ± 4.31 *
OP	72.00 ±1.13	22.95 ± 0.64 *	160.25 ± 17.12	107.12 ± 2.68	92.34 ± 1.22
ON	65.25 ± 0.92 * •	27.20 ± 1.27	170.63 ± 21.13	110.06 ± 2.67	82.06 ± 2.46
OA	66.75 ± 0.78 * •	22.65 ± 0.21 *	144.10 ± 11.32	105.81 ± 4.25	78.11 ± 6.10 *
L	73.25 ± 0.92	25.65 ± 1.06	93.98 ± 4.02	109.32 ± 3.60	83.14 ± 4.02
LP	70.45 ± 1.20	25.20 ± 0.42 *	127.03 ± 1.35	104.50 ± 1.24	88.63 ± 1.87
LN	64.55 ± 0.92 * •	26.25 ± 1.06	145.00 ± 1.37	106.21 ± 4.56	86.78 ± 1.69
LA	67.75 ± 0.07 * •	24.70 ± 0.85 *	123.21 ± 10.11	110.00 ± 3.26	89.08 ± 1.30
M	72.00 ± 1.27	29.05 ± 1.20	121.25 ± 7.32	100.36 ± 4.11	81.41 ± 3.32 *
MP	69.80 ± 1.13	26.75 ± 0.49	138.31 ± 12.35	111.21 ± 1.96	87.22 ± 9.03
MN	71.10 ± 1.13	25.45 ± 0.78 * •	171.10 ± 29.64	109.45 ± 3.97	83.86 ± 7.31
MA	70.40 ± 0.57	23.60 ± 0.85 * •	117.45 ± 23.96	113.13 ± 9.13	89.64 ± 1.65
E	74.70 ± 1.41	28.30 ± 0.99	133.08 ± 39.45	102.35 ± 1.25	90.49 ± 4.06
EP	71.15 ± 1.06	31.10 ± 0.57	119.20 ± 33.56	106.01 ± 4.78	94.12 ± 3.98
EN	65.40 ± 0.57 * •	33.45 ± 0.21 * •	141.20 ± 27.12	105.60 ± 1.95	93.30 ± 5.15
EA	69.15 ± 1.34 * •	31.15 ± 0.32	126.00 ± 25.32	107.19 ± 4.36	87.49 ± 8.23

* $P < 0.05$ compared to the respective control within columns.

• $P < 0.05$ compared to the respective intact protein within columns.

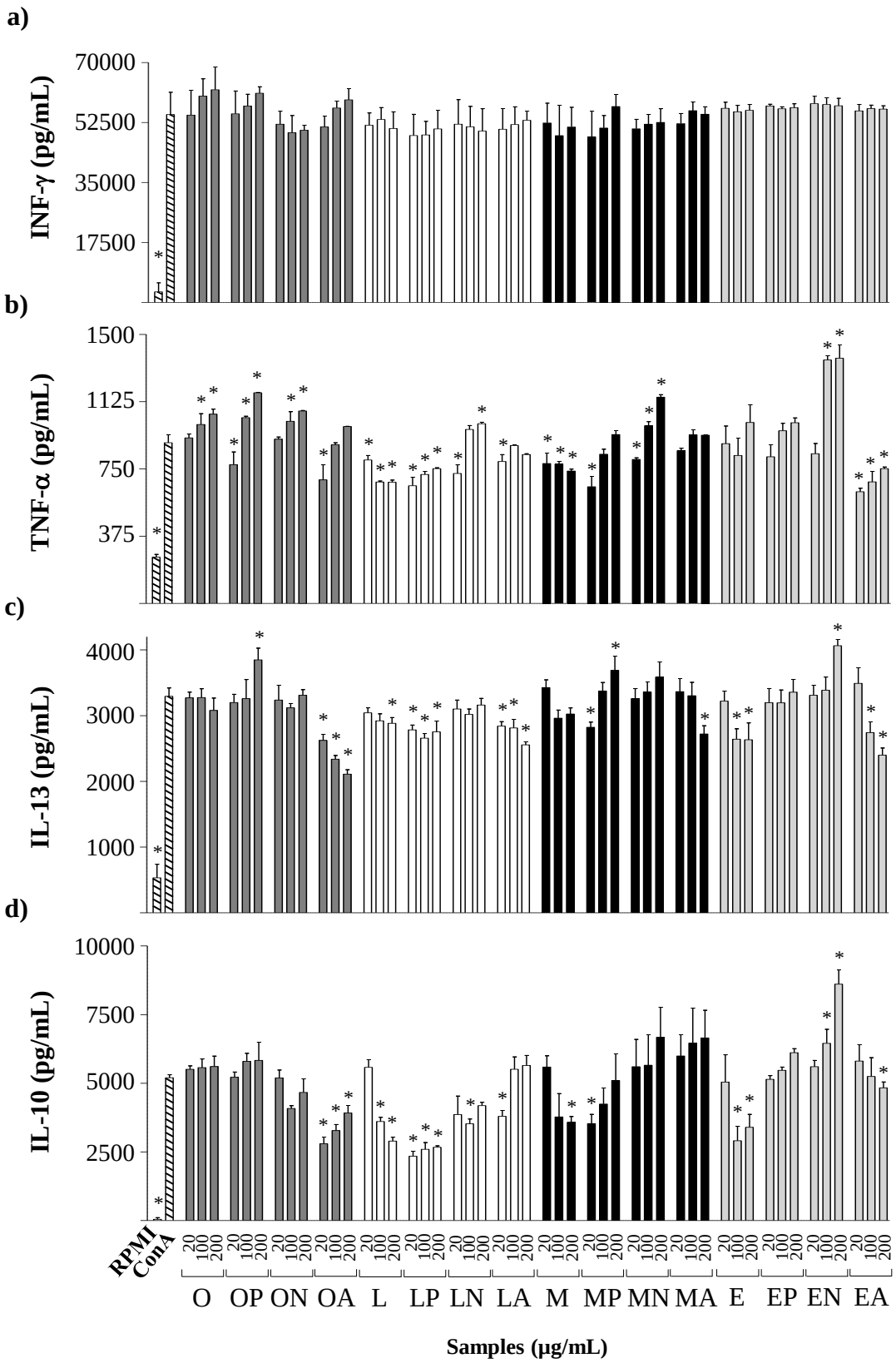

Figure 4.1.3. Effects of different concentrations (20, 100 and 200 μg/mL) of OVA (O), LYS (L), OM (M), EW (E) and their hydrolysates produced with pepsin (P), Neutrase (N), and alcalase (A) on ConA-induced secretion of IFN-γ (a), TNF-α (b), IL-13 (c), and IL-10 (d) by mouse splenocytes. Data are means ± SD of triplicates. * $P < 0.05$ compared to the ConA-stimulated control.

IL-10), similar to that produced by intact LYS (LP; Fig. 4.1.3b-d). When the effect of the highest concentration of the hydrolysates (200 μg/mL) on the proliferation of ConA-stimulated splenocytes was assessed, a significant decrease was observed after the addition of those of OVA, LYS and EW with Neutrase and alcalase, which also exerted a pronounced influence on Th1 and/or Th2 cytokines (ON, LN, EN, OA, LA and EA; Table 4.1.2). The MTT assay showed no significant effects of egg white proteins or their hydrolysates on the viability of non-stimulated, ConA- or LPS-stimulated splenocytes, ensuring that inhibition of proliferation was not due to toxicity of the hydrolysates (Table 4.1.2). It should be mentioned that the inactivated enzymes did not have any impact on proliferation or cytokine secretion by ConA-treated splenocytes, except for Neutrase, which significantly enhanced the production of TNF-α and IL-10 (Fig. 4.1.4).

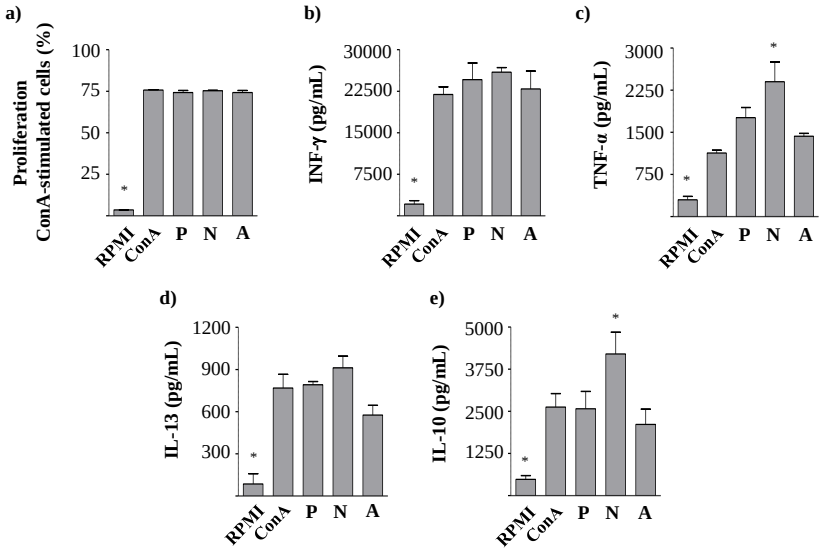

Figure 4.1.4. Effects of inactivated pepsin (P), Neutrase (N) and alcalase (A), at a concentration equivalent to that present in 200 μg/mL of the hydrolysates, on the ConA-induced proliferation (a) and secretion of IFN-γ (b), TNF-α (c), IL-13 (d) and IL-10 (e) by mouse splenocytes. Data are means ± SD of duplicates. * $P < 0.05$ compared to the ConA-stimulated control.

4.1.1.3. Egg white protein hydrolysates influence B cell responses induced by LPS and reduce oxidative stress in spleen and MLN cells

LPS was next used to stimulate murine spleen and MLN cells and estimate the effect of the addition of the egg white protein hydrolysates on antibody production and oxidative status.

LPS is an immunostimulating product of Gram-negative bacteria that signals through TLR4, acknowledged as a crucial pathogen recognition receptor in innate responses, as well as in triggering adaptive immunity (Lu et al., 2008). TLRs have been mainly identified in immune cells, especially monocytes, macrophages and DCs, as well as in other cell types, where they participate in responses associated to oxidative stress and inflammation. LPS is also as a potent mouse B lymphocyte mitogen which promotes expansion, class switch recombination and immunoglobulin secretion; and it is known that LPS-activated B cells acquire differential modulatory effects on T cell polarization (Xu et al., 2008).

LPS enhanced spleen cell proliferation (although less than ConA; Table 4.1.2) and secretion of IgG1 (Fig. 4.1.5) and IgG2a (not shown), which are, respectively, Th2- and Th1-biased antibodies, from spleen and MLN cells. All the individual egg white proteins (but not EW) and their hydrolysates with pepsin, Neutrase and alcalase, reduced the production of IgG1 by LPS-stimulated spleen cells, although the lowest levels, which were in turn significantly different from those produced by the intact proteins, were induced by the hydrolysates of OVA, LYS and OM with alcalase (Fig. 4.1.5a). Likewise, these hydrolysates, as well as that of LYS with pepsin, reduced IgG1 secretion from MLN cells (Fig. 4.1.5b). Incubation of spleen and MLN cell cultures with the egg white proteins and their hydrolysates also decreased the LPS-induced secretion of IgG2a, although, in this case, there were no significant differences between them (not shown).

The inhibiting effect of the hydrolysates produced by alcalase and that of LYS with pepsin on IgG1 secretion by LPS-stimulated spleen and MNL cells (OA, LA, MA and LP, Fig. 4.1.5) is consistent with the reduction observed in the secretion of Th2-related cytokines by the cells stimulated with ConA in the presence of these hydrolysates, indicated above (Fig. 4.1.3c). In addition, these hydrolysates also significantly decreased LPS-induced splenocyte proliferation (OA, LA, MA and LP; Table 4.1.2). Furthermore, while, in general terms, all the hydrolysates inhibited ROS generation in *t*-BOOH-stimulated spleen and MLN cells, the highest antioxidant activity corresponded to those produced by alcalase (Fig. 4.1.6). The

inactivated enzymes did not affect the proliferation or the production of IgG1 and ROS by LPS-stimulated spleen cells (Fig. 4.1.7).

When the mean values of the effects exerted by the highest concentration (200 μg/mL) of hydrolysates with pepsin, Neutrase and alcalase on cytokine and antibody secretion, oxidative stress and cell proliferation were compared, the hydrolysates of egg white proteins with alcalase differed from the hydrolysates with Neutrase ($P < 0.05$) by virtue of their decreasing effects on the production of TNF-α, IL-13, IgG1 in MLN, and ROS in splenocytes and MLN cells (Table 4.1.3).

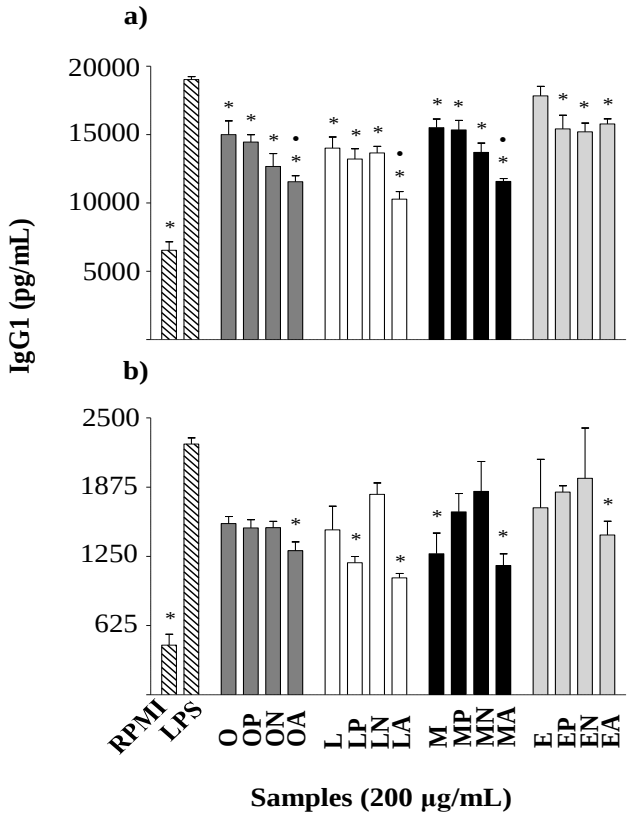

Figure 4.1.5. Effect of OVA (O), LYS (L), OM (M), EW (E) and their hydrolysates produced with pepsin (P), Neutrase (N), and alcalase (A) (200 μg/mL) on LPS-induced secretion of IgG1 by mouse splenocytes (a), and MLNs cells (b). Data are means ± SD of triplicates. * $P < 0.05$ compared to the LPS-stimulated control. • $P < 0.05$ compared to the respective intact protein.

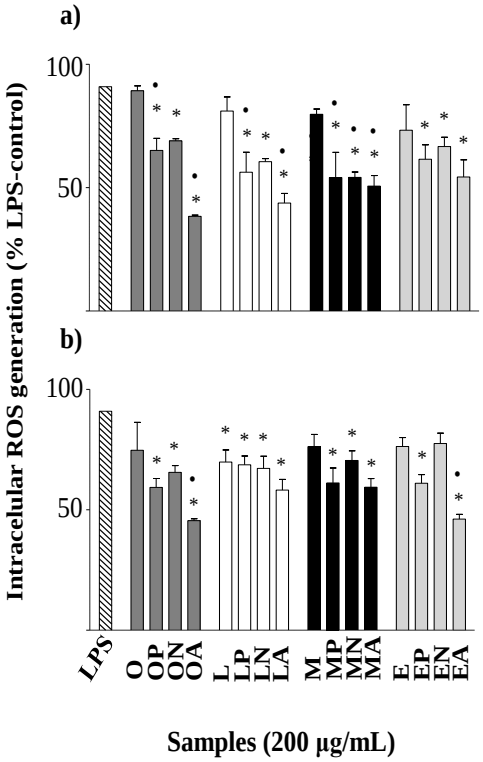

Figure 4.1.6. Effect of OVA (O), LYS (L), OM (M), EW (E) and their hydrolysates produced with pepsin (P), Neutrase (N) and alcalase (A) (200 µg/mL) on *t*-BOOH-induced intracellular ROS generation by LPS-stimulated mouse splenocytes (a), and MLN cells (b). Data are expressed as percentage of the LPS-stimulated control ± SD of triplicates. * $P < 0.05$ compared to the LPS-stimulated control. • $P < 0.05$ compared to the respective intact protein.

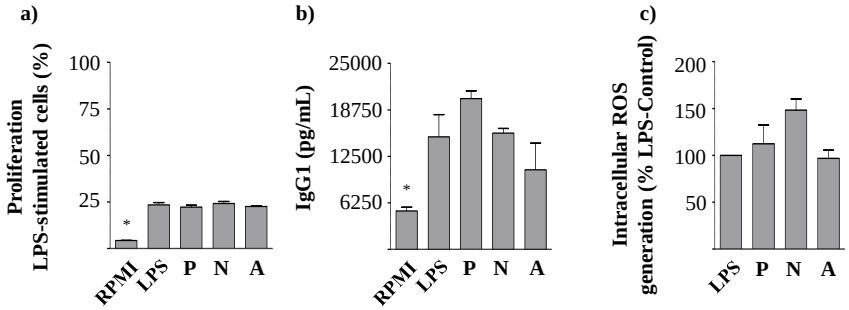

Figure 4.1.7. Effects of inactivated pepsin (P), Neutrase (N) and alcalase (A), at a concentration equivalent to that present in 200 µg/mL of the hydrolysates, on the LPS-induced proliferation (a), intracellular ROS generation (b) and secretion of IgG1 (c) by mouse splenocytes. Data are means ± SD of duplicates. * $P < 0.05$ compared to the LPS-stimulated control.

Table 4.1.3. Mean values of the effects of 200 μg/mL of the hydrolysates of OVA, LYS, OM and EW produced with pepsin, Neutrase and alcalase on cytokine and antibody secretion, ROS production and cell proliferation by splenocytes and MLN cells stimulated with ConA or LPS. In order to examine the influence of the enzyme used to produce the hydrolysates, one-way ANOVA was used, followed by the Fisher's Least Significant Difference test.

Parameter	Pepsin	Neutrase	Alcalase
IFN-γ	56414.75	52525.75	55885.75
TNF-α	969.25^{ab}	1148.00^{a}	876.50^{b}
IL-13	3412.00^{a}	3530.75^{a}	2446.75^{b}
IL-10	4929.25	6035.00	5264.25
IgG1 produced by splenocytes	14330.00	13361.50	12296.25
IgG1 produced by NLM cells	1545.50^{ab}	1772.75^{a}	1242.00^{b}
IgG2a produced by splenocytes	418.50	457.50	354.75
IgG2a produced by NLM cells	80.50	81.25	76.75
ROS produced by splenocytes	65.16^{a}	68.82^{a}	51.45^{b}
ROS produced by MLN cells	67.35^{b}	72.21^{a}	54.32^{c}
Proliferation induced by ConA	70.85^{a}	66.58^{b}	68.51^{ab}
Proliferation induced by LPS	26.50	28.09	25.53

a-b Different letters indicate significant differences ($P < 0.05$) within rows.

4.1.2. Discussion

This study shows that peptides released by enzymatic hydrolysis of egg white proteins can modulate the immune responses of murine cells to mitogen stimuli in different ways, revealing potential activities that could be used for different purposes as Th1- or Th2-skewing mediators. Considering the traditional division of the role of Th1 and Th2 responses in cell-mediated and antibody-mediated immune reactions, Th1-type immune activation involves the secretion of IFN-γ, which acts as an inductor of anti-microbial and anti-tumour defence mechanisms through the production of pro-inflammatory cytokines and cytotoxic ROS by effector cells; while Th2-type cells synthetize cytokines that help B lymphocytes to develop into certain types of IgG and IgE antibody producing cells. Both types of T cells, with the contribution of other T lymphocyte subsets, such as Th17 and T reg cells, cross-regulate each other, so that an imbalanced response leads to oxidative stress and abnormally amplified inflammatory signals, or to food allergy and asthma (Soyer et al., 2013; Becker et al., 2014).

The hydrolysates exerted effects different from those of the intact proteins from which they derived, with OVA stimulating mainly Th1 cytokines (TNF-α), LYS and OM inhibiting the release of both Th1 and Th2 cytokines (TNF-α, IL-13 and IL-10), and EW mainly inhibiting Th2 mediators (IL-13 and IL-10). The hydrolysates of OVA and LYS with alcalase reduced ConA- and LPS-stimulated murine lymphocyte proliferation, production of Th2-biased cytokines, such as IL-13 and IL-10, and, at low concentrations, also decreased the secretion of the Th1 cytokine TNF-α. In agreement with these observations, these hydrolysates considerably inhibited IgG1-class switching induced by LPS, which is part of the IL-4-mediated Th2 response, and counteracted the release of ROS induced by an oxidant agent such as *t*-BOOH. Likewise, the hydrolysate of EW with alcalase decreased ConA-induced proliferation, as well as both Th1 (TNF-α) and Th2 (IL-13, IL-10) responses and ROS generation in spleen and MNL cells challenged with *t*-BOOH, an effect similar to that exerted by the hydrolysate of LYS with pepsin that can be interpreted as immunosuppressive. In contrast, the hydrolysates of OVA, LYS and EW with Neutrase also decreased the proliferative response to ConA, but increased the level of TNF-α, leading to less pronounced antioxidant effects.

It is noteworthy that the most potent immunomodulating hydrolysates in this experimental system, those produced by Neutrase and alcalase, suppressed the mitogen-induced proliferation of lymphocytes even if they exerted distinct effects on the cytokines released. Unlike our results, other studies reported that single food peptides (Jiehui et al., 2014) or food protein hydrolysates (Mao et al., 2007; Saint-Sauveur et al., 2008; Yang et al., 2009a; Hou et al., 2012; Chalamaiah et al., 2014) promoted lymphocyte proliferation induced by ConA, which was interpreted as a stimulatory effect on cell-mediated immunity. However, those reports assessed cell viability, the number of healthy cells in a sample, rather than cell proliferation, that is, the number of dividing cells. In fact, the hydrolysates of egg white proteins assayed did not change the number of viable cells (which allows discarding possible toxic effects); despite they decreased mitogen-induced proliferation as part of their immunoregulating role. In the cells treated with the hydrolysates produced by alcalase, reduced cytokine and antibody secretion

followed the pattern of T cell proliferation, whereas in the case of those produced by Neutrase, it cannot be excluded that the enzyme preparation itself exerted an immunostimulating effect, as judged by the increased release of TNF-α and IL-10, which would have masked the influence of the peptides.

The existence of egg white protein-derived antioxidant peptides with radical scavenging activities, or able to inhibit low density lipoprotein oxidation and ameliorate the blood lipid profile and the oxidative status *in vivo* has already been described (Dávalos et al., 2004; Manso et al., 2008; Huang et al., 2010; You et al., 2010). However, while it is recognized that antioxidants, such as polyphenols or omega-3 fatty acids, can improve the immune function, to the best of our knowledge, there are no previous reports on the capacity of egg proteins hydrolysates to modulate the immune response in murine cell cultures. Furthermore, while the potential immunomodulating use of peptides has traditionally focused on their ability to prevent and repair the damage caused by oxidative stress and inflammatory reactions (Duarte et al., 2006; Nelson et al., 2007; Phelan et al., 2009; Vernaza et al., 2012; Fernández-Tomé et al., 2014; Mallet et al., 2014) by enhancing Th2-type responses (Vinderola et al., 2005; Duarte et al., 2006; Nelson et al., 2007; Yang et al., 2009a; Jiehui et al., 2014), or to stimulate the secretion of Th1 cytokines without an effect on the release of Th2 cytokines and, thus, to play a role in fighting infections (Saint-Sauveur et al., 2008; Phelan et al., 2009); the present results suggest that other uses are possible. Thus, the hydrolysates produced with alcalase, able to combine Th2-supresive properties with a remarkable antioxidant capacity, could be of interest as immunotherapeutic approaches against excessive Th2 responses and inflammatory conditions, such as those typical of food allergies (Soyer et al., 2013).

Even if it is now well known that, in addition to Th2-cytokine producing T cells and antibody-producing B cells, other cells of the innate and adaptive immunity play a role in the induction and regulation of the inflammatory processes that drive the development of allergy, the *in vitro* model system used appeared useful to evaluate the immunomodulating and antioxidant properties of the egg white protein hydrolysates and to discriminate between the

impact of those produced with various enzymes on effector lymphocyte activation. It is well documented that hydrolysates of the same protein produced with different enzymes differ in the immunological response they generate in cultured cells (Phelan et al., 2009; Chalamaiah et al., 2014); even though, due to the complex peptide composition of most food protein hydrolysates, it is expected that they would exert multiple effects through different mechanisms (Samaranayaka and Li-Chan, 2011). As compared to the hydrolysates produced by pepsin and Neutrase, those produced by alcalase did not show distinct features regarding their RP-HPLC profile, albeit they had a smaller molecular mass distribution. We then decided to conduct further studies to correlate particular physicochemical characteristics with the immunomodulatory properties of egg white protein-derived peptides, as well as to further validate these results in more sophisticated *in vitro* models.

.

4.2. Hydrolysates of egg white proteins regulate exacerbated immune responses in human cells *in vitro*

Among the features of food proteins that play a role in their capacity to induce an allergic response, it is recognized their ability to promote the differentiation and expansion of Th2 cells that secrete IL-4, IL-5 and IL-13, favouring Th2 rather than Th1 immunity (Berin and Shreffler, 2008). Accordingly, immunomodulating peptides that lack IL-4 inducing capacity but hold IFN-γ stimulating properties, able to re-establish the Th1/Th2 balance, stand as an interesting option for allergy prevention and treatment (Yang et al., 2010; Wei et al., 2016).

Nevertheless, despite the apparent opposite effects of Th2 and Th1 immunity, there are examples of antioxidant and anti-inflammatory compounds that decrease the effects mediated by IFN-γ and the intracellular pathways that accelerate the generation of ROS (Zaknun et al., 2012), but also reduce the expression of Th2 cytokines and IgE levels in response to food allergens in mice (Yano et al., 2007; Zuercher et al., 2010; Masilamani et al., 2011). In this respect, previous results (section 4.1) showed that certain enzymatic hydrolysates of egg white proteins could not only inhibit the release of Th2- (IL-13) and Th1-cytokines (TNF-α), but also reduce the production of IgG1 and the generation of ROS in unspecifically stimulated murine cells.

With the aim to go further in the evaluation of the anti-allergenic potential of the egg white protein hydrolysates we assessed their ability to deviate the unbalanced immune status representative of food allergy. Thus, the immunomodulating potential of the hydrolysates was estimated as their ability to hinder, on the one hand, cytokine and IgE production by Th2-skewed human PBMCs and, on the other hand, the release of pro-inflammatory factors and ROS generation from PBLs subjected to a Th1 stimulus (Fig. 4.2.1). The binding to IgE of egg allergic patients was determined and the peptides present in the hydrolysates were identified, as a contribution to the characterization of hydrolysates that combine the ability to stimulate T lymphocytes and modulate the immune response with a reduced capacity to bind IgE and, eventually, trigger clinical symptoms.

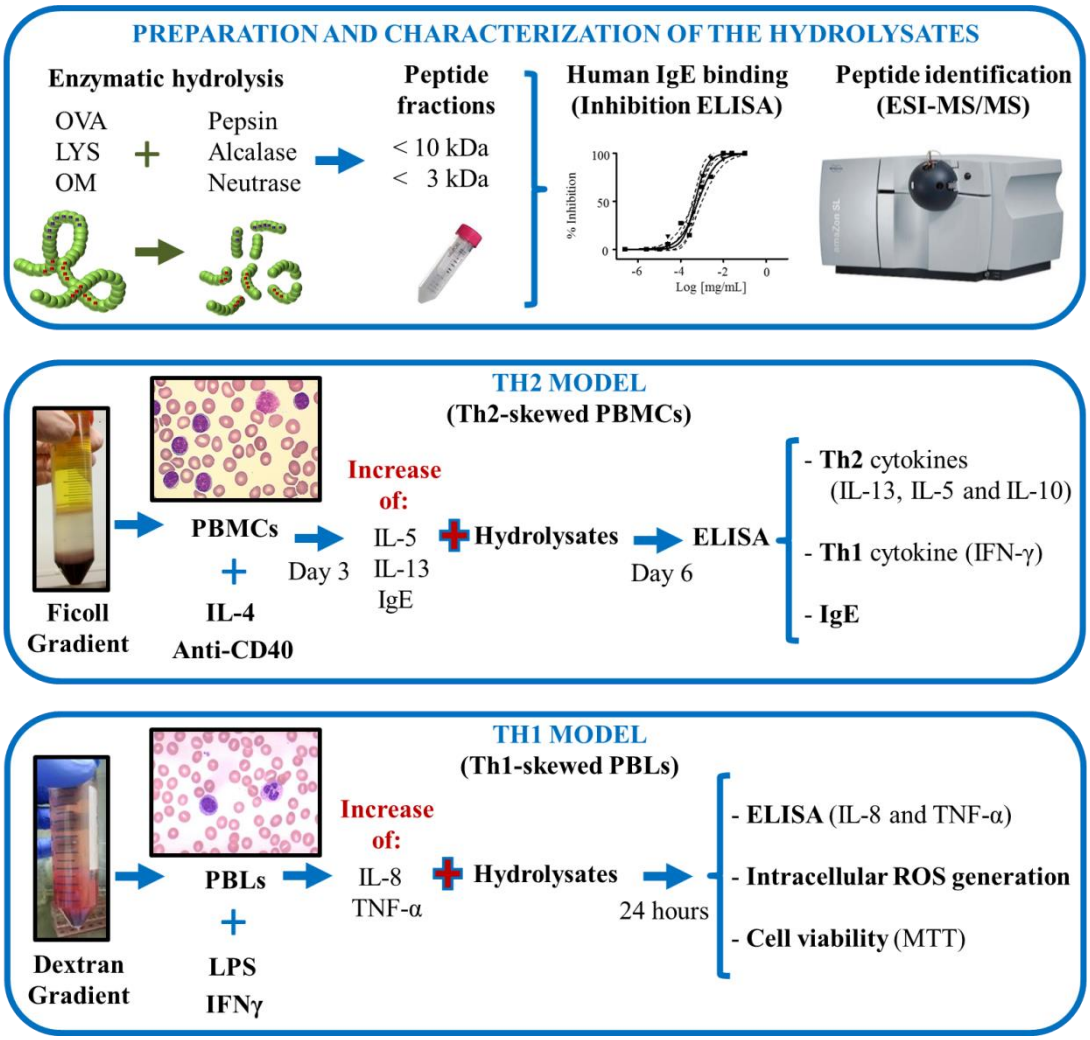

Figure 4.2.1. Scheme of the experimental procedure followed in section 4.2. ESI-MS/MS, electrospray ionization mass/mass; LPS, lipopolysaccharide; LYS, lysozyme; MTT, method based on 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazoliumbromide; OM, ovomucoid; OVA, ovalbumin; PBLs, peripheral blood leukocytes; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; Th, T-helper cell.

4.2.1. Results

4.2.1.1. The hydrolysates of egg white proteins with pepsin and alcalase inhibit the release of Th2 cytokines and IgE from Th2-primed PBMCs

PBMCs form 6 non-allergic donors, previously selected from a total of 8 volunteers on the basis of a positive induction of Th2 cytokines (IL-5 and IL-13) with anti-CD40 and IL-4, released IL-13 (187.05 ± 53.42 pg/mL), IL-5 (78.16 ± 19.24 pg/mL), IFN-γ (125.41 ± 25.63 pg/mL) and IL-10 (69.36 ± 10.21 pg/mL), following culture with these molecules for 3 days.

Levels of IL-13, IL-5 and IL-10 were significantly ($P < 0.05$) higher than those corresponding to the cells cultured with IMDM alone (60.95 ± 29.17, 7.55 ± 4.50 and 17.32 ± 10.87 pg/mL, respectively), while those of IFN-γ were significantly lower (IMDM: 296.80 ± 24.84 pg/mL), thus reflecting a shift to an augmented Th2/Th1 cytokine profile. In view of the variability of the results, the cytokine levels were normalized to those induced by anti-CD40 and IL-4 in each of the 6 selected donors (100%). As shown in Fig. 4.2.2, stimulation with LPS significantly down regulated the production of Th2 cytokines (IL-13 and IL-5), and up regulated the release of the Th1 cytokine IFN-γ and that of IL-10.

The hydrolysates of OVA and OM with pepsin and alcalase exerted a dose-dependent inhibition of the release of IL-13 and IL-5 induced by previous anti-CD40 and IL-4 conditioning, which was significant, particularly at the highest doses (Figs. 4.2.2a and b). Likewise, stimulation with the hydrolysate of LYS with alcalase significantly reduced the levels of IL-13 and IL-5 in a dose-dependent manner, although it should be noted that intact LYS also produced a Th2-inhibitory effect similar to that exerted by this hydrolysate. The hydrolysates of egg white proteins with alcalase did not significantly change the secretion of the Th1 cytokine IFN-γ, except for the hydrolysate of LYS with alcalase at 100 μg/mL; while the hydrolysates of OVA, LYS and OM with pepsin at 100 and 200 μg/mL stimulated the release of IFN-γ (Fig. 4.2.2c).

Conversely, the hydrolysates of OVA and OM with Neutrase increased IL-13 and IL-5 and that of LYS with Neutrase increased IL-13 (Figs. 4.2.2a and b). In general terms, these hydrolysates also stimulated the production of IFN-γ (Fig. 4.2.2c) and, therefore, they could be regarded as inducers of both Th2 and Th1 responses. Furthermore, the production of IL-10 was significantly enhanced by the hydrolysates of OVA, LYS and OM with Neutrase at the highest concentration (200 μg/mL).

Conditioning of PBMCs with anti-CD40 and IL-4 induced cells to produce IgE. As shown in Fig. 4.2.3, incubation with LPS and with the hydrolysates of OVA and OM with

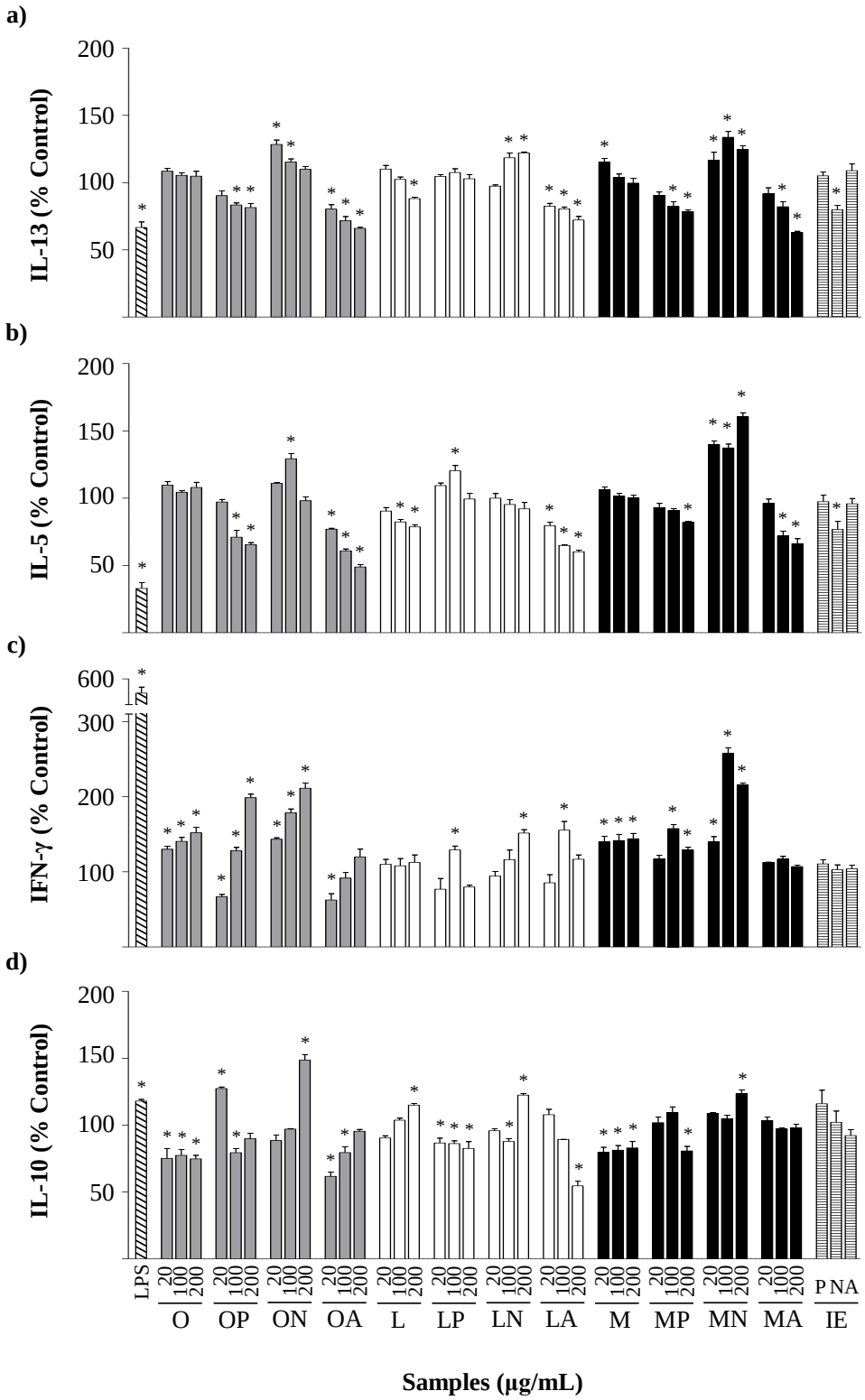

Figure 4.2.2. Effects of different concentrations (20, 100 and 200 µg/mL) of OVA (O), LYS (L), OM (M), and their hydrolysates with pepsin (P), Neutrase (N) and alcalase (A), as well as the inactivated enzymes (IE) at a concentration equivalent to that present in 200 µg/mL of the hydrolysates, on the secretion of IL-13 (a), IL-5 (b), IFN-γ (c) and IL-10 (d) by Th2-skewed PBMCs. Data are expressed as percentage of the values induced by anti-CD40 and IL-4 ± SEM in 6 donors stimulated in triplicate and * indicates significant differences ($P < 0.05$) compared to this control (100%).

pepsin and OVA, LYS and OM with alcalase, that resulted in reduced secretion of IL-5 and IL-13 (Figs. 4.2.2a and b), also inhibited IgE production by primed B cells. Of note is the observation that the hydrolysates of LYS with pepsin enhanced the production of IgE, while those of egg white proteins with Neutrase did not change it, despite, in general terms, they increased Th2 cytokines.

The inactivated enzymes did not have any impact on cytokine or IgE secretion, except for Neutrase, which inhibited IL-13 and IL-5 production by 20 and 33%, respectively (Figs. 4.2.2 and 4.2.3). On the other hand, the MTT assay, carried out in order to assess the cytotoxicity of the stimulation with anti-CD40 and IL-4, as well as that of the proteins and their hydrolysates at a concentration of 200 µg/mL, allowed discarding significant effects on cell viability (Fig 4.2.4).

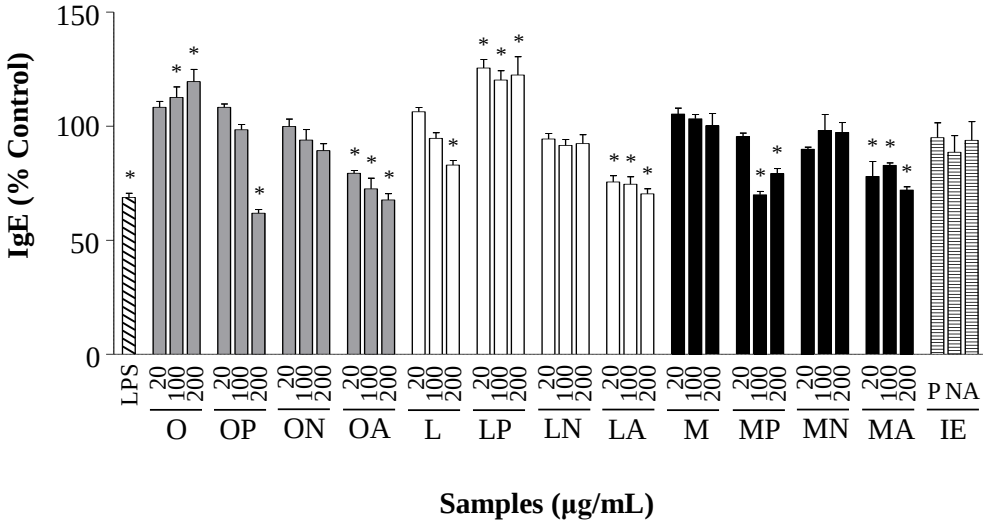

Figure 4.2.3. Effects of different concentrations (20, 100 and 200 µg/mL) of OVA (O), LYS (L), OM (M), and their hydrolysates with pepsin (P), Neutrase (N) and alcalase (A), as well as the inactivated enzymes (IE) at a concentration equivalent to that present in 200 µg/mL of the hydrolysates, on the secretion of IgE by Th2-skewed PBMCs. Data are expressed as percentage of the values induced by anti-CD40 and IL-4 ± SEM in 6 donors stimulated in triplicate and * indicates significant differences ($P < 0.05$) compared to this control (100%).

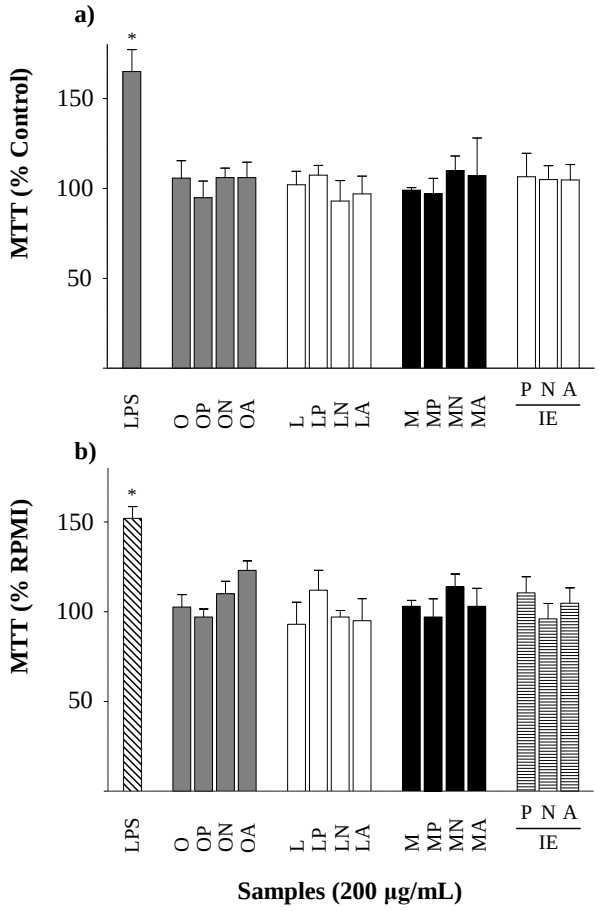

Figure 4.2.4. Effects of OVA (O), LYS (L), OM (O), their hydrolysates with pepsin (P), Neutrase (N) and Alcalase (A) (200 µg/mL), and the inactivated enzymes (IE) at a concentration equivalent to that present in 200 µg/mL of the hydrolysates, on cell viability, determined by the MTT method, of Th2-skewed PBMCs (a) and non-stimulated PBLs (b). Data are expressed as percentage of the values induced by IL-4 and anti-CD40 (a) or of the non-stimulated cells (b) ± SEM. * indicates significant differences ($P < 0.05$) compared to their respective controls (100%).

4.2.1.2. The hydrolysates of egg white proteins with pepsin inhibit the release of pro-inflammatory mediators and the hydrolysates with alcalase hinder ROS generation from Th1-primed PBLs

PBLs from 8 donors were previously selected from a total of 10 volunteers on the basis of a positive induction of an inflammatory response on stimulation with LPS and IFN-γ, which was characterized by the release of mediators such as TNF-α (1048.23 ± 495.12 *vs* 14.59 ± 8.26 pg/mL in RPMI incubated cells, $P < 0.05$) and IL-8 (38467.95 ± 9479.32 *vs* 986.11 ± 321.86 pg/mL in RPMI incubated cells, $P < 0.05$).

As shown in Fig. 4.2.5, OVA increased the production of TNF-α and IL-8, while the hydrolysates of LYS and OM with pepsin reduced the secretion of TNF-α; and those of OVA, LYS and OM with pepsin, and LYS with alcalase, reduced the secretion of IL-8 (Figs. 4.2.5a and b). In addition and, in general terms, the hydrolysates of the three proteins with pepsin and alcalase neutralized ROS generation by *t*-BOOH (Fig. 4.2.5c). Inactivated pepsin and alcalase did not affect the production of cytokines or ROS, but Neutrase significantly stimulated the release of TNF-α and IL-8 (Fig. 4.2.5). Cytotoxicity assessment with MTT did not reveal any significant adverse effects of LPS or the hydrolysates at the highest concentration (200 μg/mL) on PBLs viability (Fig. 4.2.4).

Since most antioxidant peptides derived from food sources have molecular mass from 0.5 to 1.8 kDa (Samaranayaka and Li-Chan, 2011), and considering that the best results, in terms of inhibition of the release of pro-inflammatory mediators and oxidative stress in PBLs, were obtained in the cells treated with the hydrolysates of the three egg white proteins with pepsin and alcalase, these were fractionated to assess the contribution of peptides with molecular mass lower than 10 and 3 kDa. The results obtained (Figs. 4.2.5d, e and f) suggest that, in general terms, small peptides were responsible for the anti-inflammatory and antioxidant effects observed. The fractions of OVA and LYS with pepsin decreased the secretion of TNF-α by, approximately, 50% (Fig. 4.2.5d) and, overall, the reduction in the generation of IL-8 and ROS was more marked when cells were incubated with the fractions as compared with the complete hydrolysates (Figs. 4.2.5e and f). However, the fractions derived from the hydrolysates of OM with pepsin exerted a less pronounced effect on all the parameters measured.

4.2.1.3. The hydrolysates with pepsin and Neutrase are recognized by human IgE and contain IgE-binding epitopes

We next examined the binding of the hydrolysates to IgE from sera of egg allergic patients (Table 4.2.1). The highest IgE-binding corresponded to OVA, followed by LYS and

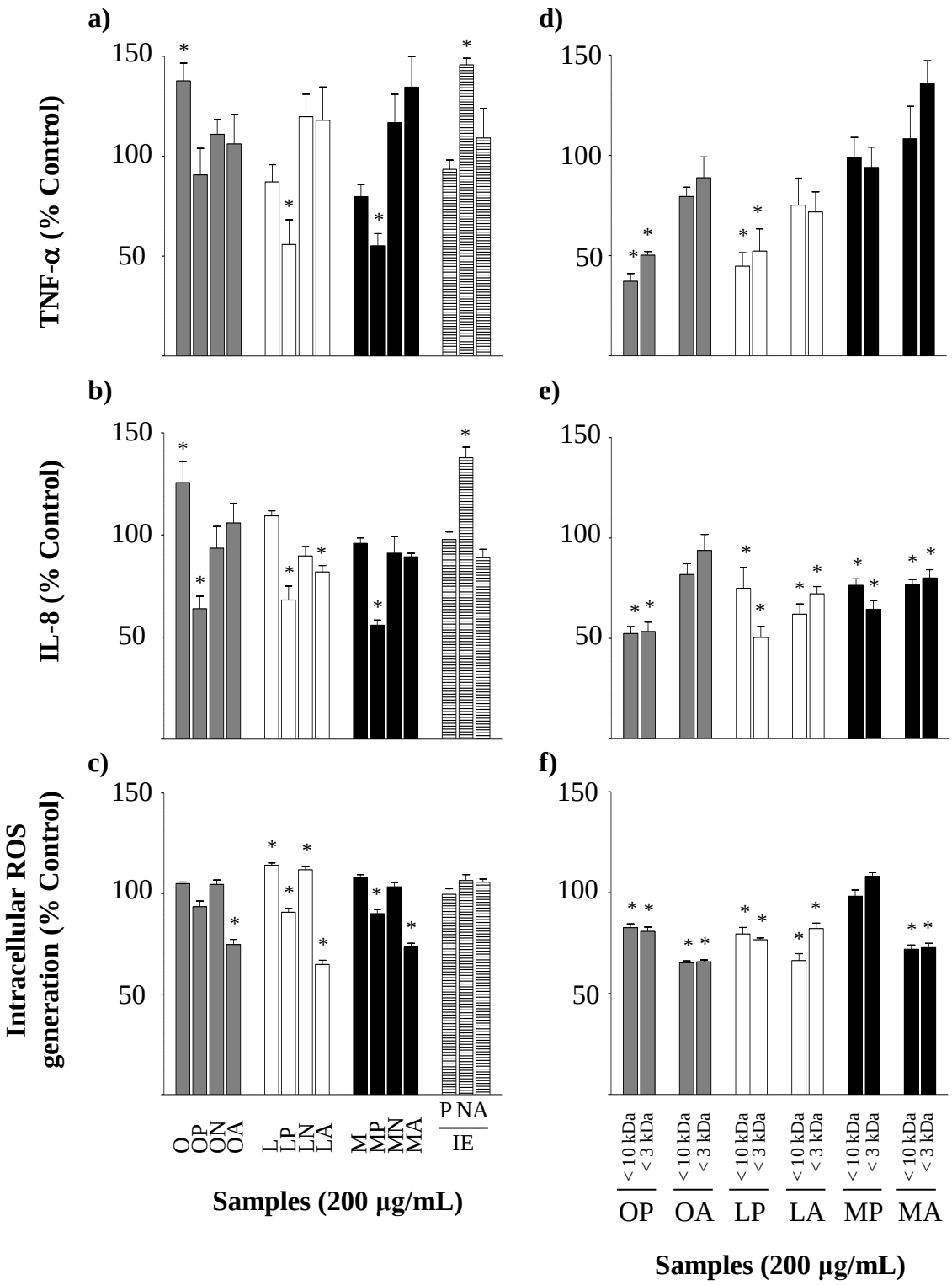

Figure 4.2.5. Effects of OVA (O), LYS (L), OM (M), their hydrolysates with pepsin (P), Neutrase (N) and alcalase (A) (200 µg/mL), the inactivated enzymes (IE) at a concentration equivalent to that present in 200 µg/mL of the hydrolysates, and the fractions with molecular mass lower than 10 and 3 kDa on the secretion of TNF-α (a, d) and IL-8 (b, e), and intracellular ROS generation (c, f) by Th1-skewed PBLs. Data are expressed as percentage of the values induced by IFN-γ and LPS ± SEM in 8 donors stimulated in triplicate and * indicates significant differences ($P < 0.05$) compared to this control (100%).

OM. The hydrolysates of OVA, LYS and OM with pepsin maintained, respectively, 10, 41 and 14% of the original IgE-binding and that of OVA with Neutrase, 30%. The IgE binding of the hydrolysates of OVA with alcalase and LYS and OM with Neutrase and alcalase was negligible. RP-HPLC ruled out the existence of intact protein that could account for the residual IgE-binding of the hydrolysates (Fig. 4.1.2).

Table 4.2.1. Binding to IgE from sera of egg allergic patients of OVA (O), LYS (L), OM (M) and their hydrolysates with pepsin (P), Neutrase (N) and alcalase (A). Results are means of, at least, 3 independent experiments and are expressed as EC50, that is, the effective sample concentration (μg/mL) for 50% of the maximum binding to IgE.

	EC_{50} (μg/mL)
O	2.83 ± 0.23^{a}
OP	28.57 ± 0.50^{a}
ON	9.43 ± 0.81^{a}
OA	2113.5 ± 70.00^{b}
L	12.81 ± 2.81^{a}
LP	27.80 ± 0.05^{a}
LN	1850.00 ± 60.36^{b}
LA	851.12 ± 31.32^{c}
M	14.62 ± 0.27^{a}
MP	193.93 ± 19.73^{b}
MN	3070.51 ± 75.85^{c}
MA	2295.34 ± 36.95^{d}

a-d Different superscripts indicate significant differences ($P < 0.05$) for each protein (O, L or M).

The peptides present in the hydrolysates of OVA, LYS and OM were identified by RP-HPLC-MS/MS (Figs. 4.2.6, 4.2.7 and 4.2.8, respectively). In the hydrolysates of OVA with pepsin, Neutrase and alcalase, 140, 104 and 109 peptides were identified, respectively (Fig. 4.2.6). The hydrolysate of OVA with pepsin only had 11 peptides in common with the hydrolysate with Neutrase, and 12 with the hydrolysate with alcalase, while the hydrolysates with Neutrase and alcalase shared 10 common sequences. Several of the peptides identified in the hydrolysates of OVA with pepsin and Neutrase are comprised within previously described high frequency IgE-binding areas of the protein, such as 125-176, 188-198, 326-336 and 370-

385 (Benedé et al., 2014), which could explain their residual IgE-binding. Of note is the observation that the hydrolysate of OVA with pepsin contained peptides with free radical-scavenging properties, such as YRGGLRPINF, YQIGL, FRADHPFL and RADHPFL [OVA (125-134), OVA (212-216), OVA (358-365) and OVA (359-365)] (Dávalos et al., 2004). FRADHPFL was also found the hydrolysate of OVA with Neutrase and RADHPFL in the hydrolysate of OVA with alcalase.

In the hydrolysates of LYS with pepsin, Neutrase and alcalase, we identified 96, 95 and 107 peptides, respectively, that covered the whole LYS sequence, with only 5-8 peptides in common among the different hydrolysates (Fig. 4.2.7). As compared with the hydrolysates with Neutrase and alcalase, the hydrolysate of LYS with pepsin contained larger peptides within the reported IgE-binding areas: 11-27, 57-83 and 108-122 (Jiménez-Saiz et al., 2014). Furthermore, many of the fragments had sulfhydryl groups, and since RP-HPLC-MS/MS analyses were performed in the presence of a reducing agent (DTT) to aid identification, it is likely that disulphide-linked fragments contributed to the IgE-binding. Finally, several peptides in the hydrolysate of LYS with alcalase matched sequences previously identified in radical-scavenging fractions, such as KRHGLDNYRGY, KRHGLDNY, HGLDNY, and GLDNYRGY [LYS (13-23), LYS (13-20), LYS (15-20) and LYS (16-23)] (You et al., 2010). The hydrolysate of LYS with Neutrase contained the defence peptide IVSDGNGMNAW [LYS (98-108)] (Mine et al., 2004).

In the hydrolysates of OM with pepsin, Neutrase and alcalase, 84, 89 and 86 peptides were identified, respectively, after disulphide bond reduction with DTT, with very few common sequences (3-5 peptides) (Fig. 4.2.8). There are numerous IgE-binding epitopes distributed along the OM structure and also many differences in epitope recognition among different egg allergic patients (Järvinen et al., 2007). The observation that, comparatively, longer peptides were found in the hydrolysate of OM with pepsin and that some of them could be linked by disulphide bonds indicates that they could give rise to longer IgE-binding sequences and thus, result in a higher recognition by human IgE.

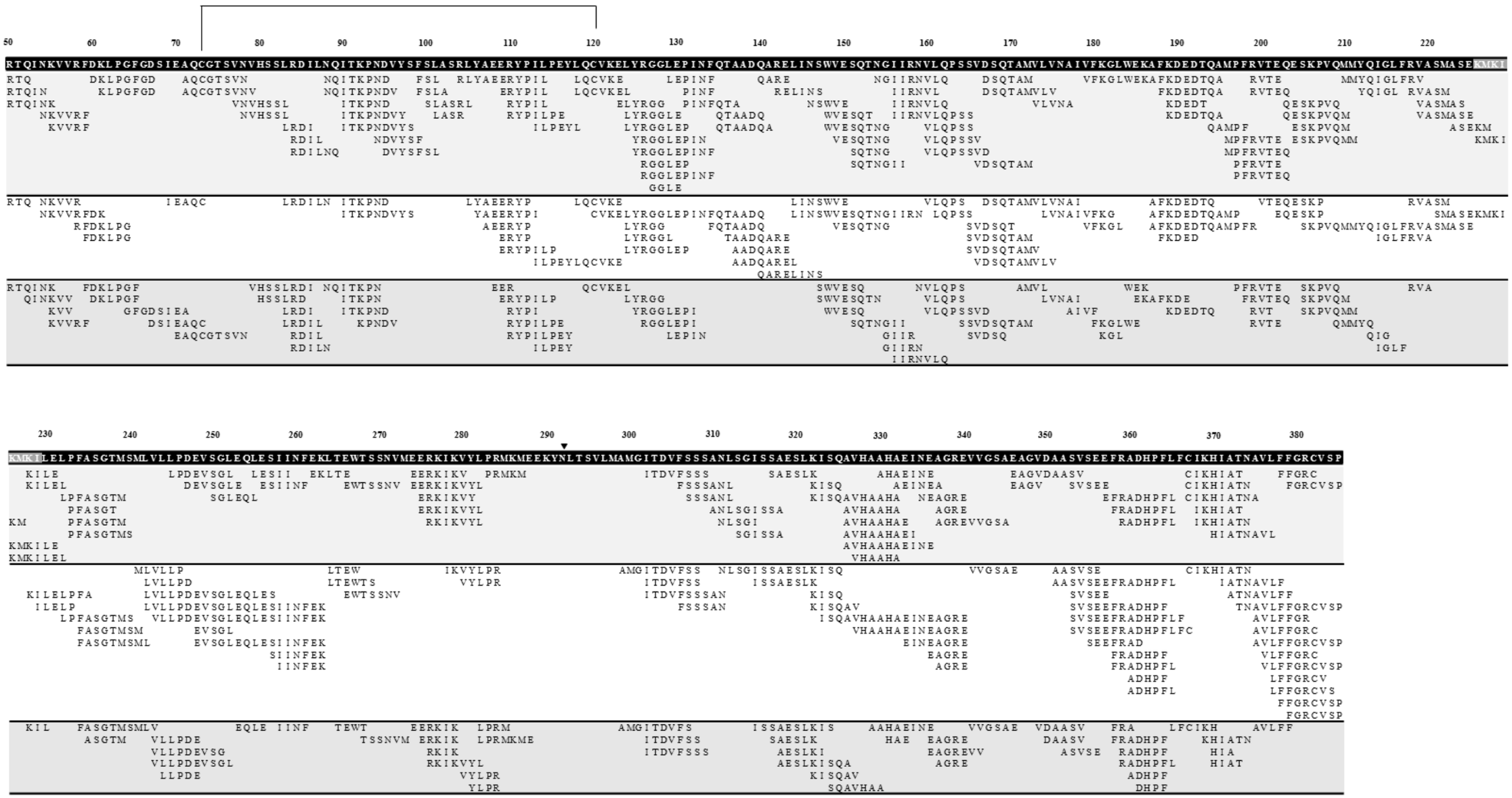

Figure 4.2.6. . Peptide sequences, identified by RP-HPLC-MS/MS in the hydrolysates of OVA with pepsin (light grey), Neutrase (white) and alcalase (dark grey). ▼: Carbohydrate chain. ⌜⌝: Disulphide bond.

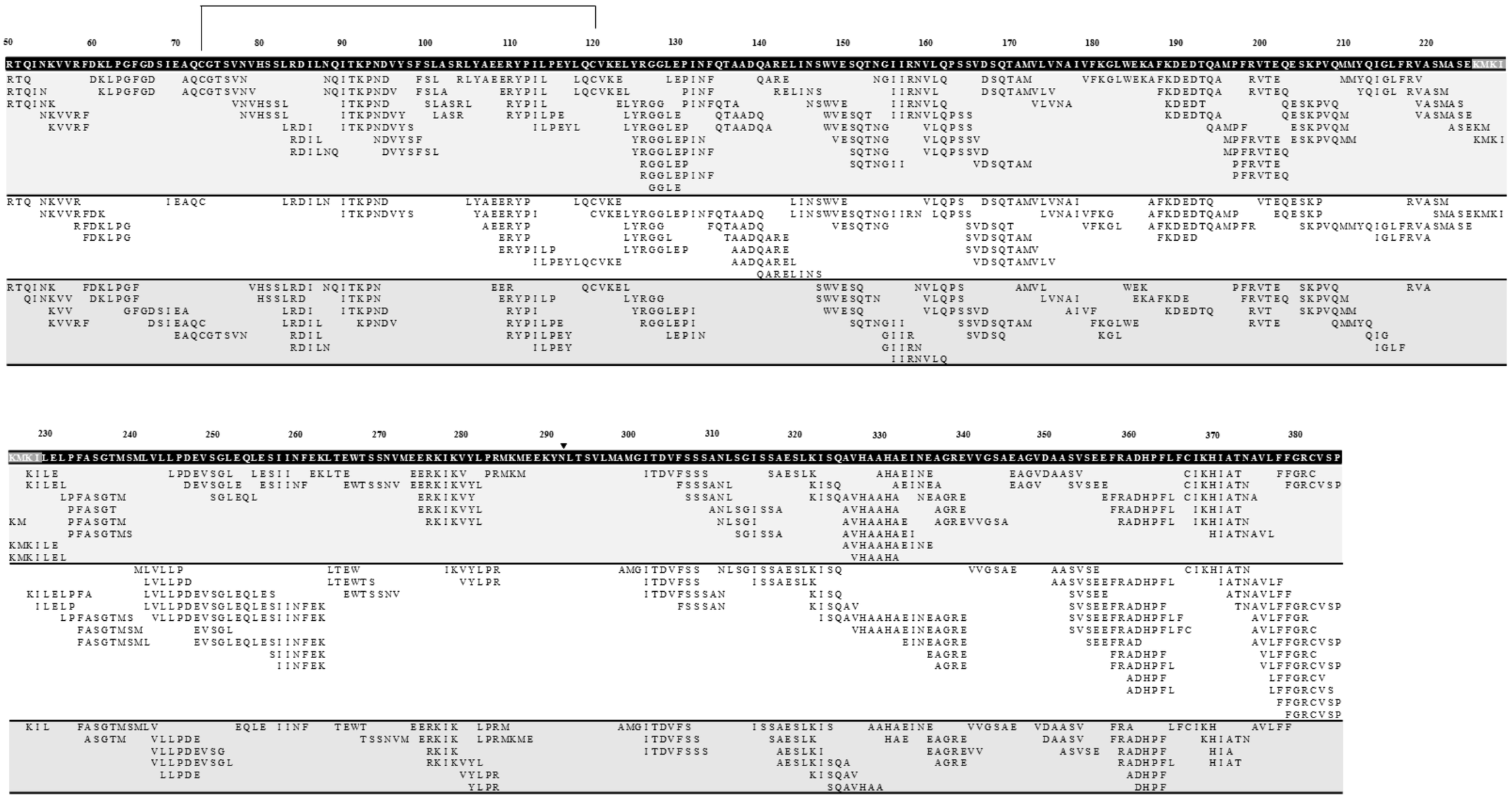

Figure 4.2.7. . Peptide sequences, identified by RP-HPLC-MS/MS in the hydrolysates of LYS with pepsin (light grey), Neutrase (white) and alcalase (dark grey). ⌜⌝: Disulphide bond.

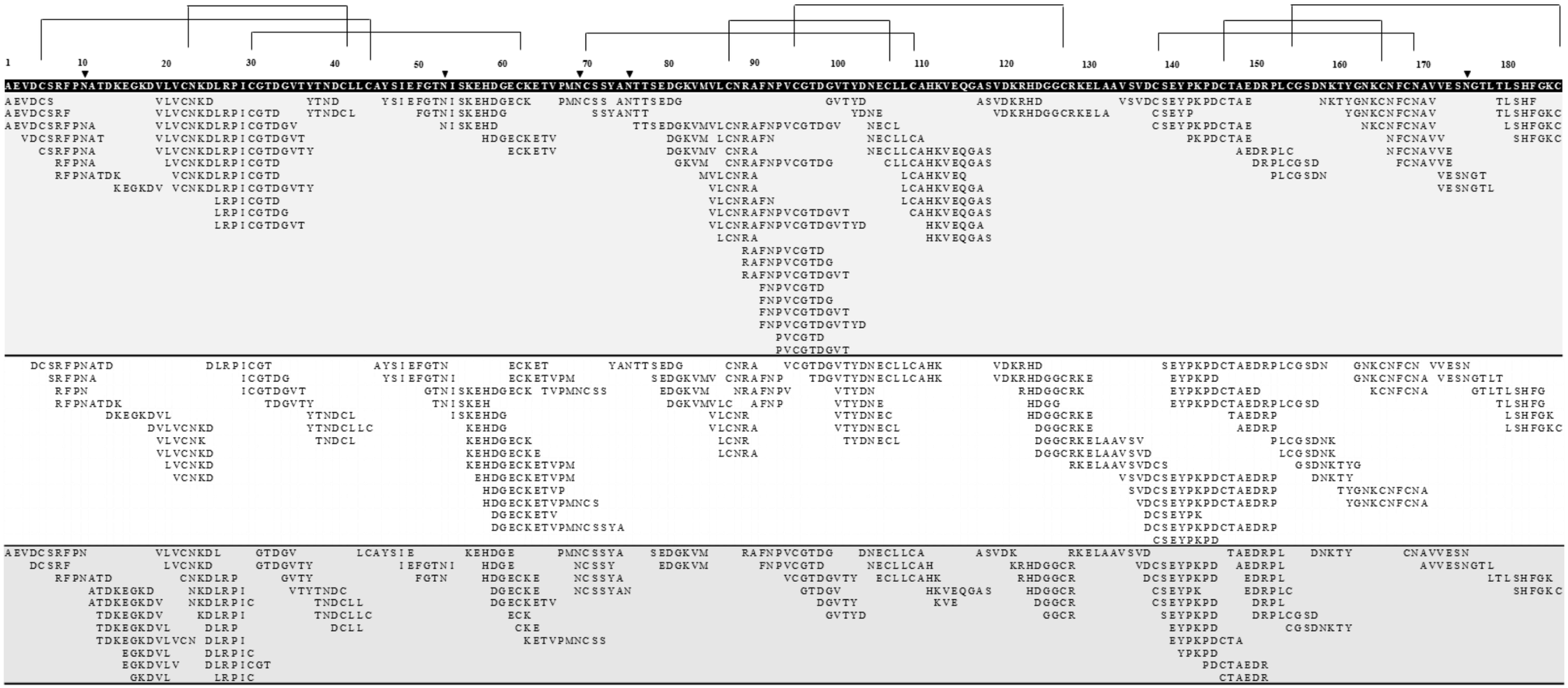

Figure 4.2.8. . Peptide sequences, identified by RP-HPLC-MS/MS in the hydrolysates of OM with pepsin (light grey), Neutrase (white) and alcalase (dark grey). ▼: Carbohydrate chain. ⌜⌝: Disulphide bond.

4.2.2. Discussion

Following a validated non-antigen specific model (Holvoet et al., 2013), a Th2-skewed cytokine profile was induced in human PBMCs from non-allergic donors. Incubation with anti-CD40 and IL-4 led to the production of IL-13, IL-5 and IL-10, and reduced the secretion of IFN-γ in most, but not all, of the donors assayed, and at different concentrations. Similarly, Holvoet et al. reported that the levels of IL-5 were highly donor dependent, although, unlike our results, these authors did not find either IFN-γ or IL-10 production after incubation with IMDM or with anti-CD40 and IL-4 (Holvoet et al., 2013). PBMCs conditioned with anti-CD40 and IL-4 not only imitate allergen-specific cells in the cytokine profile, but also in the secretion of IgE antibodies, because anti-CD40 induces the proliferation of resting B cells and IL-4 co-stimulates anti-CD40 B cell-activation and production of IgE (Rousset et al., 1991). As expected, culture with LPS reduced the secretion of IL-13 and IL-5 and increased that of IFN-γ, while it also inhibited the release of IgE. Therefore, it redirected the Th2 state in this cell model (Holvoet et al., 2013).

The hydrolysates of egg white proteins with alcalase effectively down-regulated the production of Th2 cytokines and the secretion of IgE to the culture media of Th2-skewed PBMCs. The hydrolysates with pepsin produced a similar effect, with the exception of the hydrolysate of LYS with pepsin, which did not reduce IL-5 and IL-13 production and enhanced the secretion of IgE. It has been demonstrated that the inhibition of IgE production by plasma cells exerted *in vitro* by anti-inflammatory substances correlates *in vivo* (in a mouse model of peanut allergy) with suppression of IgE and decreased adverse reactions and plasma histamine levels (López-Expósito et al., 2011). Therefore, protein hydrolysates with the ability to prevent IgE secretion by B cells might impair the availability of this antibody for participation in mass cell activation, offering protection against the development of anaphylactic symptoms.

Our results show that the hydrolysates of OVA, LYS and OM with alcalase combined *in vitro* neutralization of an excessive Th2 response and reduction of IgE secretion with a low

grade cytokine-stimulating role, which may correlate with an anti-allergic protection *in vivo* due to a homeostatic effect (Holvoet et al., 2013). On the other hand, the hydrolysates of OVA and OM with pepsin helped to re-establish the Th1/Th2 balance in the Th2-skewed cells, as they simultaneously decreased the production of IL-5 and IL-13 and increased that of IFN-γ. It should be noted that both the inhibition of allergen-induced Th1 and Th2 cytokine responses (Yang et al., 2009b), as well as the promotion of Th1-biased responses to a detriment of Th2 ones (Yang et al., 2010), have been associated to the therapeutic effects of egg white peptides in mouse models of allergy.

We next evaluated the anti-inflammatory and antioxidant properties of the hydrolysates on cells of the peripheral blood. For this purpose, we used PBLs, that contain, in addition to lymphocytes and monocytes (PBMCs), polymorphonuclear cells, which participate, together with macrophages, in the initiation and progression of inflammatory processes once being recruited to the required sites (Barnes, 2011). In order to induce inflammatory responses, PBLs were stimulated with LPS and IFN-γ, which, in agreement with previous results, led to the production of TNF-α and IL-8 (Richard et al., 2005). The hydrolysates of LYS and OM with pepsin inhibited the release of TNF-α and IL-8 in PBLs treated with inflammatory stimuli and also reduced oxidative stress in cells in which ROS generation was induced by *t*-BOOH treatment. This suggests their potential to counteract the inflammatory damage that may arise in response to allergic injuries. In turn, the hydrolysates of OVA, LYS and OM with alcalase did not avoid the release of pro-inflammatory mediators, but significantly neutralized the oxidative stress induced by *t*-BOOH. It is likely that, in these hydrolysates, except in that of OM with pepsin, peptides with molecular mass lower than 10 kDa were responsible for the anti-inflammatory and antioxidant effects observed. Pepsin digestion of OM gives rise to large fragments linked by disulphide bonds (Benedé et al., 2013) and, therefore, the content of free low molecular mass peptides in this hydrolysate is probably small.

Due to the cross-regulatory interaction between Th1 and Th2 immunity, it is hypothesized that the suppression of Th1 responses by antioxidant compounds, while beneficial

in reducing the side effects of the inflammatory processes, may lead to an up-regulation of Th2-type cytokines that promotes allergic sensitization and exacerbates allergic symptoms (Zaknun et al., 2012). However, in our experiments, we did not observe an inverse regulation of cytokine production in the Th2- and Th1-skewed cell models. The hydrolysates of OVA and OM with pepsin and the hydrolysates of OVA, LYS and OM with alcalase concurrently down-regulated features characteristic of exacerbated Th2 and Th1 responses, which suggests their potential for allergy treatment. Conversely, the hydrolysates of OVA, LYS and OM with Neutrase tended to promote both Th2 and Th1 activation in the Th2-skewed model and they could not attenuate the induction of inflammatory mediators in the Th1-skewed model. In any case, it cannot be excluded that the heat-inactivated Neutrase preparation exerted an immunomodulating effect that would have biased the influence of the egg white protein derived peptides, particularly on the Th1-primed cells.

The results obtained in these *in vitro* model systems based on human peripheral blood cells artificially skewed towards exacerbated Th2 and Th1 responses basically agree with those obtained in mitogen stimulated murine lymphocytes (section 4.1) in the case of the hydrolysates produced with alcalase and Neutrase, but not in the case of those produced with pepsin. Overall, the present cell models allowed a more precise estimation of the influence of the hydrolysates on the cytokine profiles that modulate allergic responses with the use of accessible human cells. It should be mentioned that, probably because of the low frequency of allergen-specific T cells, previous attempts to assess the immunomodulatory effects of egg proteins on PBMCs from egg allergic patients were unsuccessful due to the lack of sufficient stimulation leading to cytokine and antibody secretion (results not shown). In this respect, it is worth noting that, although allergen-specific T cell lines have been used to overcome the frequency limitation, *in vitro* expansion can alter cell phenotypes or bias the results through the selection of the rapidly proliferating clones (Pascal et al., 2013).

IgE-binding was comparatively higher in the hydrolysates with pepsin (as well as in the hydrolysate of OVA with Neutrase), which was related to the length of the peptides formed and

the existence of disulphide linked fragments that could carry bivalent epitopes. The low IgE-binding of the hydrolysates with alcalase could be attributed to the fact that they contained short peptides, with molecular masses lower than 1.5 kDa. Pepsin digestion has already been shown to reduce the reactivity of IgE from human sera to egg white proteins, such as OVA (Benedé et al., 2014), LYS (Jiménez-Saiz et al., 2014), and OM (Benedé et al., 2013). Indeed, pepsin hydrolysis of fish and nut allergens effectively down-regulates the allergic responses they cause in sensitized humans and mice (Untersmayr et al., 2007; Kulis et al., 2012). The results presented in this section show that the hydrolysates of OVA and OM with pepsin modulated Th2 cytokines and IgE secretion, while attenuating inflammatory responses. Comparatively, the hydrolysates of OVA, LYS and OM with alcalase also equilibrated Th2 differentiation efficiently, but exhibited a more reduced IgE-binding. Therefore, it can be hypothesized that these hydrolysates would elicit milder allergic reactions while retaining T cell-stimulating abilities, which might correlate with an anti-allergic benefit.

4.3. Hypoallergenic hydrolysates re-equilibrate egg white allergen responses induced *ex vivo* in cells from sensitized mice

The results obtained so far (sections 4.1 and 4.2) showed that the hydrolysates of OVA and OM with pepsin helped to re-establish the Th1/Th2 balance in Th2-biased PBMCs, while they also inhibited the release of pro-inflammatory mediators and reduced oxidative stress in PBLs treated with inflammatory stimuli. In turn, the hydrolysates of OVA, LYS and OM with alcalase, inhibited Th2 differentiation and ROS generation in mitogen stimulated murine lymphocytes and peripheral blood cells treated, respectively, with Th2 or Th1-biasing mediators. In view of their potential to modulate different phases of the allergic response, we assessed the *in vivo* allergenicity of these hydrolysates by means of PCA tests, and explored the possibility that they could modulate cytokine responses to egg allergens *ex vivo*, using splenocytes from BALB/c mice sensitized to individual egg proteins or to their mixtures in different proportions (Fig. 4.3.1). Finally, the best candidate for peptide-based IT was fractionated, in order to enrich the immunostimulating and immunomodulating peptides, and the sequences contained in each fraction were identified by mass spectrometry in an attempt to correlate peptide structure and immunoregulatory function (Fig. 4.3.1).

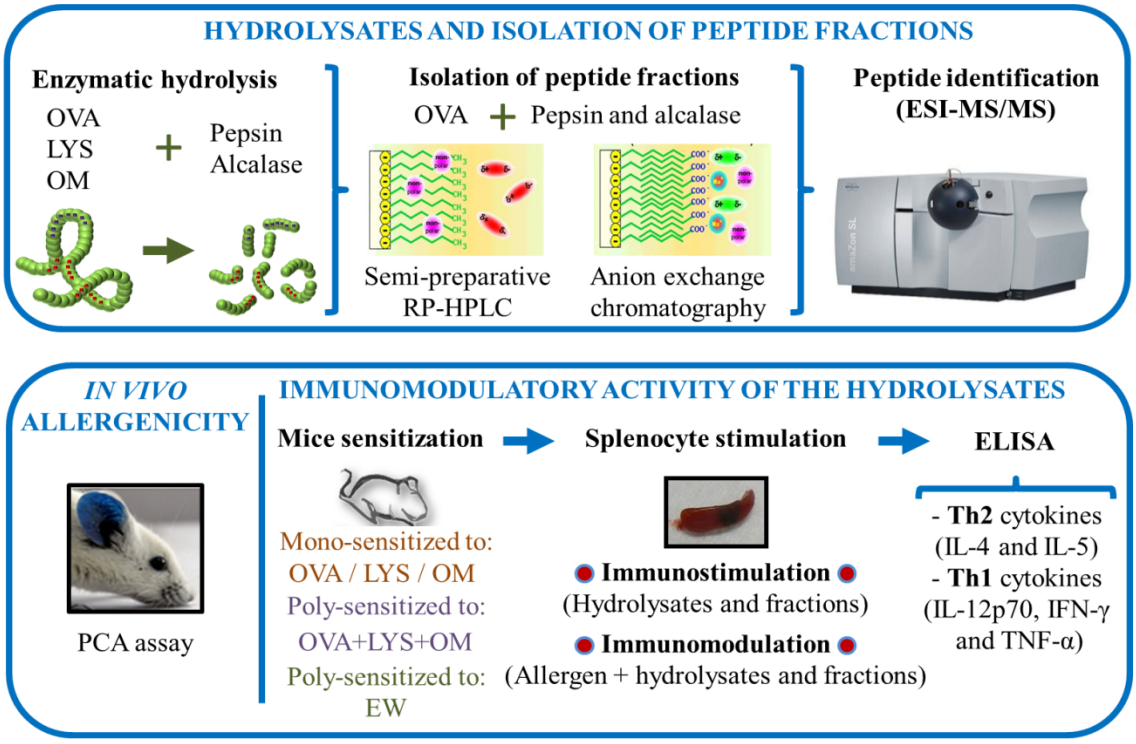

Figure 4.3.1. Scheme of the experimental procedure followed in section 4.3. ESI-MS/MS, electrospray ionization mass/mass; LYS, lysozyme; OM, ovomucoid; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis assay; RP-HPLC, reverse phase high-performance liquid chromatography.

4.3.1. Results

4.3.1.1. The hydrolysate of OVA with pepsin and the hydrolysates with alcalase do not elicit allergic responses in vivo

The ability of the egg white hydrolysates to trigger cutaneous allergic reactions was assessed *in vivo* using PCA tests with sera of mice mono-sensitized to OVA, LYS or OM (Fig. 4.3.2). Table 4.3.1 shows the concentrations of specific antibodies of the sera used for passive sensitization. Intravenous challenge of mice with the respective allergens caused a significant extravasation, as measured by the localized release of Evans blue into the tissues, in the ears injected with sera from sensitized mice compared with the control ears, injected with sera from naïve mice (Fig. 4.3.2).

In contrast with the corresponding intact protein, dye extravasation was significantly lower in mice challenged with the hydrolysate of OVA with pepsin and with the hydrolysates produced with alcalase, which indicates a diminished eliciting capacity (Fig. 4.3.2). However, the hydrolysates of LYS and OM with pepsin caused, respectively, a local reaction similar or even higher than that of the allergenic protein from which they derive.

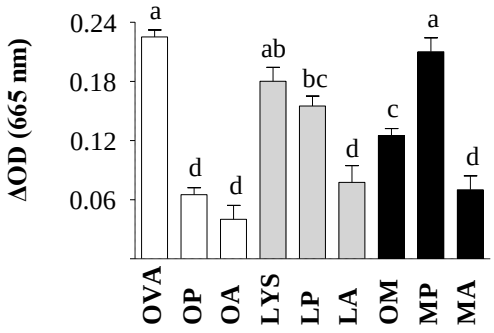

Figure 4.3.2. Allergenicity of OVA (O), LYS (L), OM (M) and their hydrolysates with pepsin (P) and alcalase (A) assessed by PCA assays. Allergen-specific dye extravasation was calculated by correcting the allergen or hydrolysate induced absorbance with the absorbance of the supernatant of the ear injected with pooled naïve sera. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

Table 4.3.1. Levels of specific IgE and IgG1 antibodies against OVA, LYS and OM determined by ELISA in sera from mice individually sensitized to the respective egg white allergen and used for PCA assays.

Sensitization	Specific IgE (ng/mL)	Specific IgG1 (ng/mL)
Naïve	0.00 ± 0.00	0.00 ± 0.00
OVA	1020.22 ± 192.98	10667.98 ± 2517.54
LYS	54.04 ± 12.39	36566.83 ± 5567.85
OM	869.48 ± 1146.33	29006.36 ± 1036.86

4.3.1.2. The hydrolysates of OVA and LYS with pepsin and alcalase show a low Th2-stimulating activity in spleen cells from sensitized mice

Spleen cells from mice sensitized to OVA, LYS or OM were stimulated with the protein used in each case for sensitization and its hydrolysates with pepsin and alcalase at various concentrations. As expected, incubation with the sensitizing protein induced a significant dose-dependent release of the Th2 cytokines IL-4 (Fig. 4.3.3a-c) and IL-5 (Fig. 4.3.4a-c). OVA and LYS, unlike OM, also increased the production of the Th1 cytokine TNF-α, although no clear dose-effect relationships were apparent (Fig. 4.3.5a-c).

In general terms, hydrolysis of OVA and LYS with pepsin reduced their Th2-stimulating capacity, while pepsin treatment did not affect the OM-induced secretion of IL-4 and IL-5. Hydrolysis of OVA, LYS and OM with alcalase abrogated their ability to stimulate Th2 responses in spleen cells, yielding Th2 cytokine levels equivalent to those of the negative control (RPMI) (Figs. 4.3.3a-c and 4.3.4a-c). The hydrolysates of OVA with pepsin and alcalase, at the highest concentrations (150 and 200 μg/mL), enhanced the production of TNF-α by the splenocytes compared with the intact protein, whereas no major differences were found between LYS, OM and their hydrolysates regarding TNF-α secretion.

Very similar results, concerning Th2 responses, were found in the splenocytes from mice poly-sensitized to the same amount of the three proteins, as well as in those from mice sensitized to EW (Figs. 4.3.3d-i and 4.3.4d-i). With respect to Th1 cytokines, the hydrolysates of OVA, LYS and OM induced a lower production of TNF-α by the splenocytes from EW-

sensitized mice than the proteins from which they derive (Fig. 4.3.5d-i). To discard an immunostimulating influence of the inactivated enzymes, their effect on splenocytes from EW-sensitized mice was evaluated at the maximum concentration used in the hydrolysates and no significant differences were observed in the production of the Th2 and Th1 cytokines studied as compared with the negative control (Table 4.3.2).

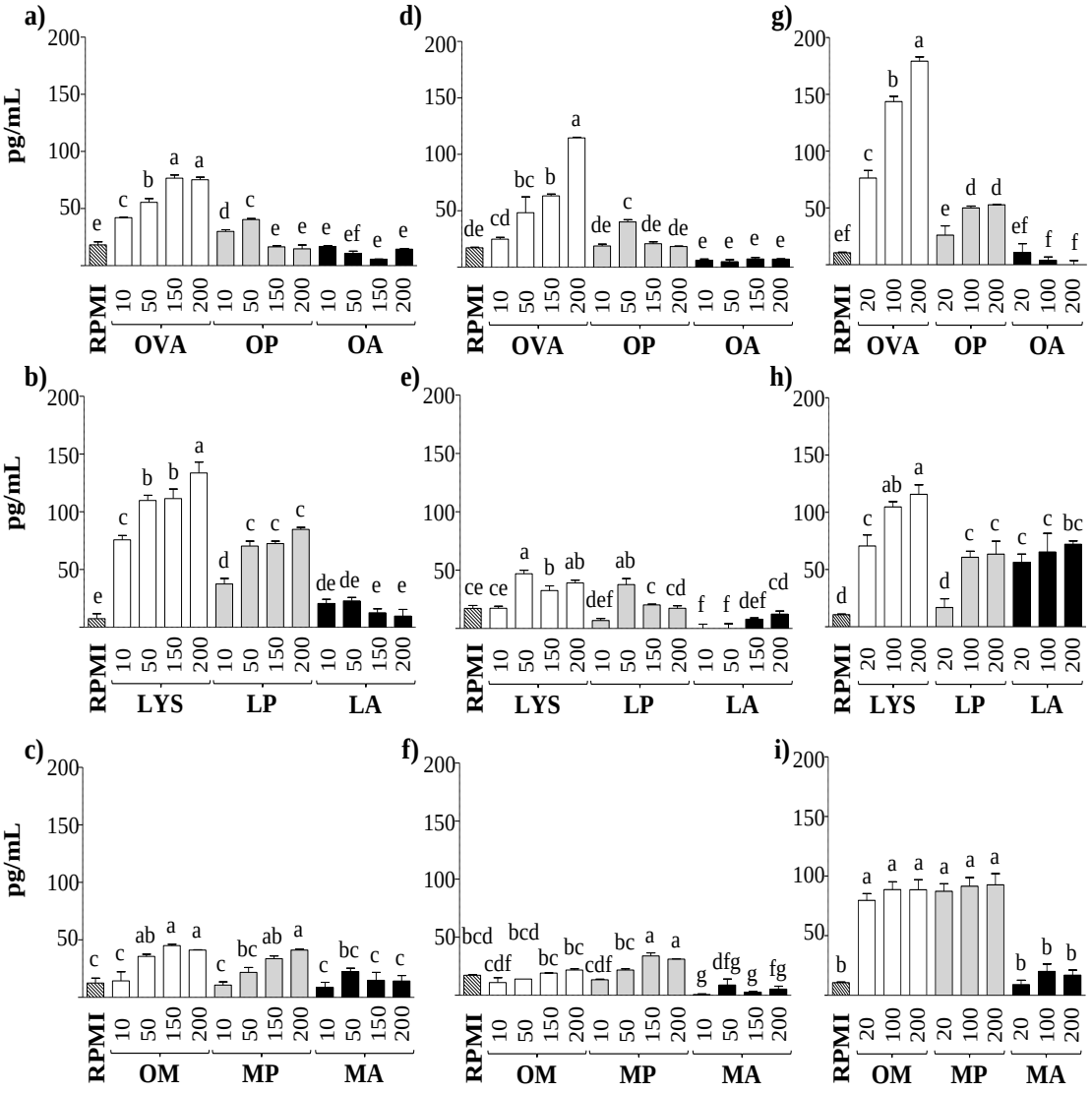

Figure 4.3.3. Immunostimulating effects of different concentrations (from 10 to 200 μg/mL) of OVA (O), LYS (L), OM (M) and their hydrolysates with pepsin (P) and alcalase (A) on the secretion of IL-4 by splenocytes from mono-sensitized (a, b and c), poly-sensitized (d, e and f) and EW-sensitized (g, h and i) mice. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

Table 4.3.2. Effects of inactivated pepsin and alcalase, at a concentration equivalent to that present in 200 μg/mL of the hydrolysates, on the secretion of IL-4, IL-5, TNF-α and IFN-γ by splenocytes from EW-sensitized mice. The secretion induced by EW is also shown for comparison. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$) for each cytokine.

	RPMI	Pepsin	Alcalase	EW
IL-4	10.6 ± 0.5^{b}	0.8 ± 1.3^{b}	0.4 ± 0.7^{b}	127.9 ± 13.0^{a}
IL-5	18.9 ± 2.7^{b}	14.3 ± 2.3^{b}	15.1 ± 3.3^{b}	161.7 ± 6.0^{a}
TNF-α	3.4 ± 0.3^{b}	7.1 ± 3.3^{b}	4.3 ± 2.3^{b}	118.1 ± 10.3^{a}
IFN-γ	17.2 ± 0.7^{b}	17.6 ± 25.8^{b}	31.6 ± 54.2^{b}	1777.4 ± 65.0^{a}

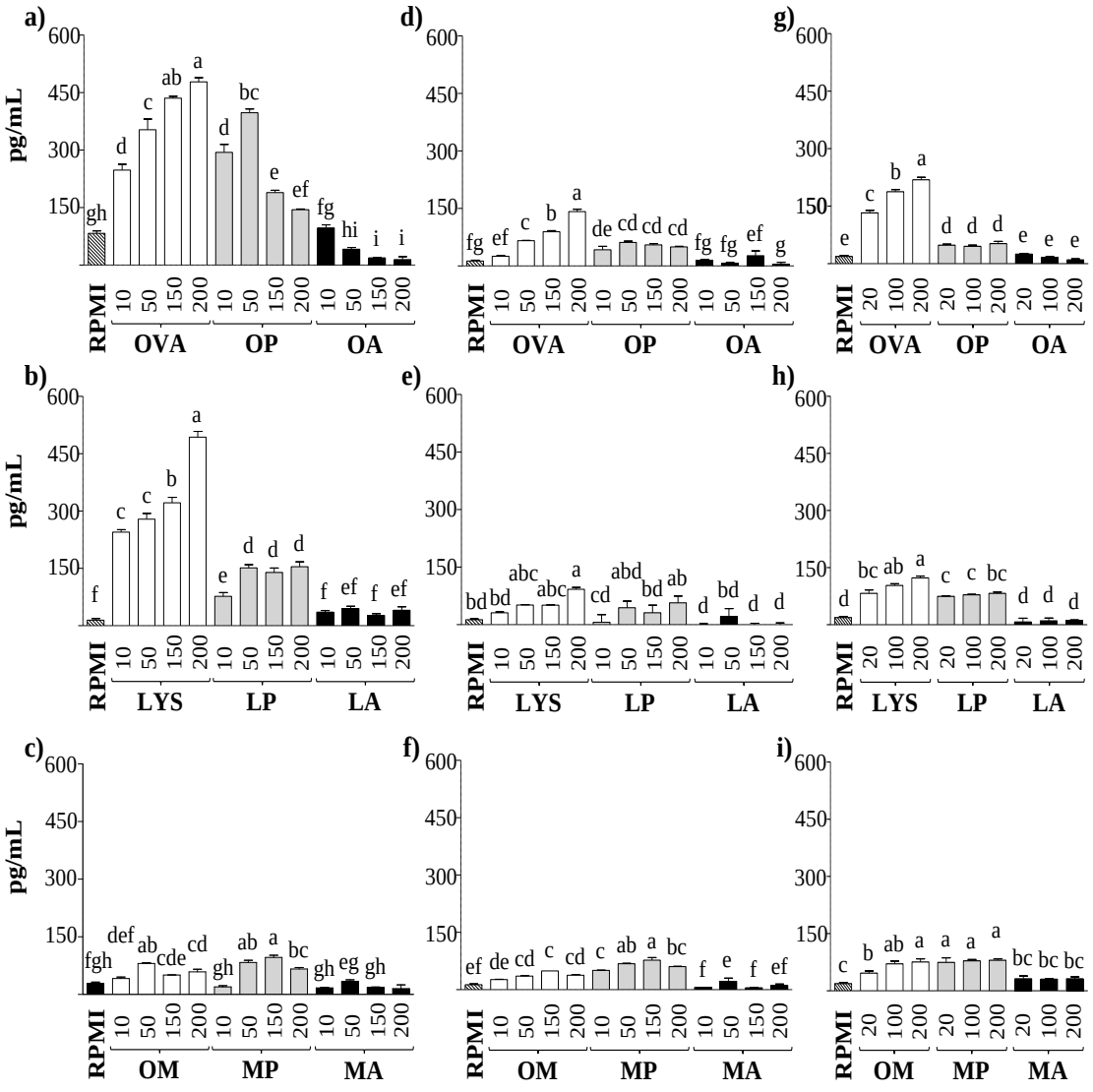

Figure 4.3.4. Immunostimulating effects of different concentrations (10, 50, 150 or 200 μg/mL) of OVA (O), LYS (L), OM (M) and their hydrolysates with pepsin (P) and alcalase (A) on the secretion of IL-5 by splenocytes from mono-sensitized (a, b and c), poly-sensitized (d, e and f) and EW-sensitized (g, h and i) mice. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

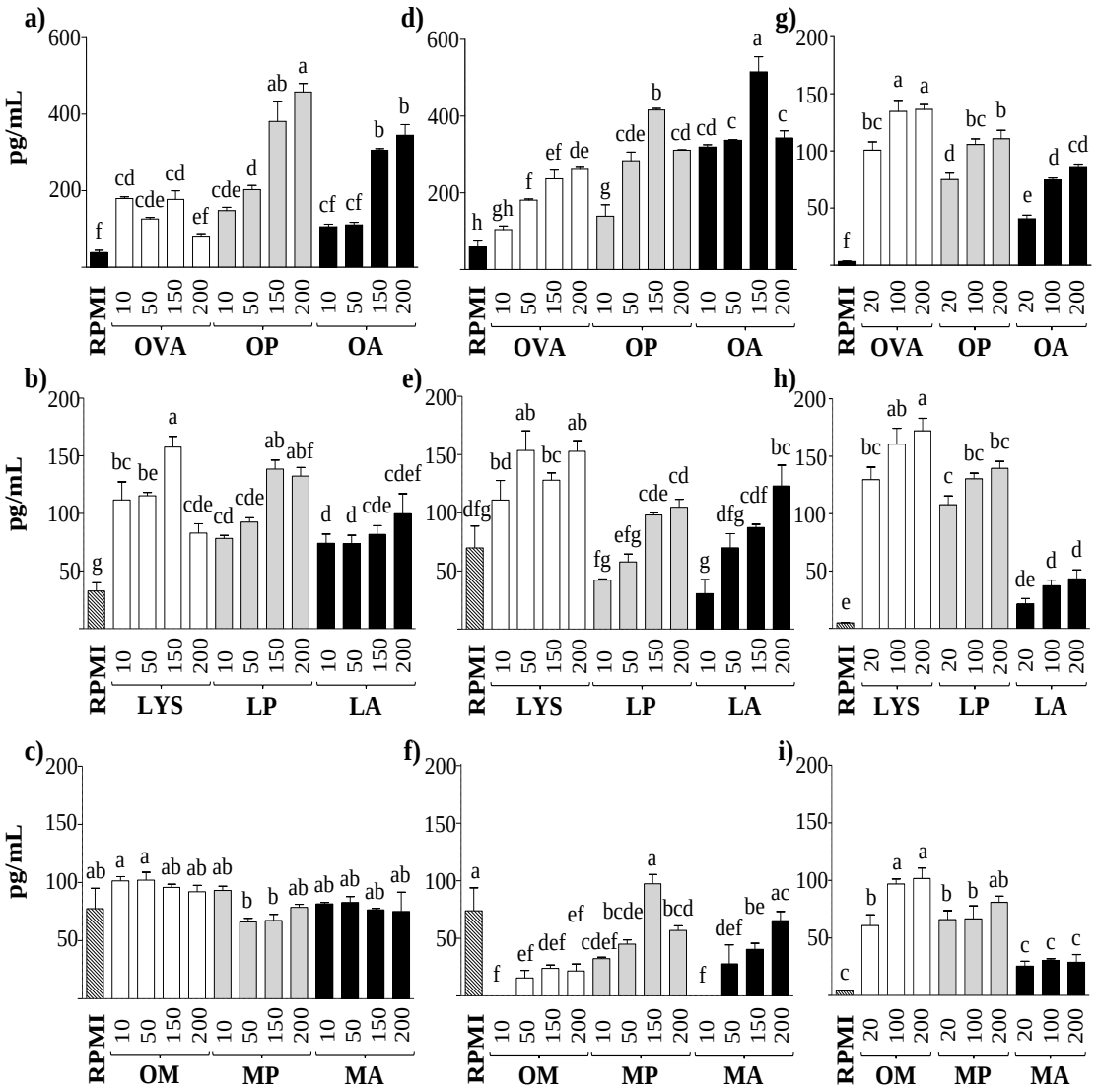

Figure 4.3.5. Immunostimulating effects of different concentrations (from 10 to 200 μg/mL) of OVA (O), LYS (L), OM (M) and their hydrolysates with pepsin (P) and alcalase (A) on the secretion of TNF-α by splenocytes from mono-sensitized (a, b and c), poly-sensitized (d, e and f) and EW-sensitized (g, h and i) mice. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

Overall, our results indicate that the hydrolysates of OVA and LYS with pepsin and alcalase promoted a less pronounced *ex vivo* Th2 activation of spleen cells from sensitized mice than the respective intact proteins, with comparable outcomes in simple (mono-sensitized) and complex (poly-sensitized or EW-sensitized) models of egg allergy. This prompted us to investigate whether they could down-regulate the allergic responses induced by the allergens from which they derive.

4.3.1.3. The hydrolysate of OVA with pepsin re-equilibrates immune responses induced by egg allergens on spleen cells from sensitized mice

In order to test whether the allergen-induced responses of immune cells from sensitized mice could be modulated by the hydrolysates, splenocytes were stimulated with the allergens used for sensitization in the presence of their corresponding hydrolysates at a concentration of 200 μg/mL of each substance, and the impact on the secretion of Th2 and Th1 cytokines was determined. OVA-induced production of IL-4 and IL-5 was inhibited by co-incubation of spleen cells from both OVA-sensitized and poly-sensitized mice with the hydrolysates of OVA with pepsin and alcalase ($P < 0.05$, Table 4.3.3).

Table 4.3.3. Immunomodulating effects of the hydrolysates of OVA (O), LYS (L) and OM (M) with pepsin (P) and alcalase (A). Splenocytes from mono- and poly-sensitized mice were stimulated with the allergen (200 μg/mL) in the absence or presence of the hydrolysates (200 μg/mL), and the secretion of IL-4, IL-5 and TNF-α was evaluated. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P< 0.05$) among each allergen and its hydrolysates for each cytokine and type of sensitization.

		Mono-sensitized mice			**Poly-sensitized mice (OVA+LYS+OM)**		
		IL-4	IL-5	TNF-α	IL-4	IL-5	TNF-α
OVA		75.3 ± 2.2^{a}	478.1 ± 10.9^{a}	81.3 ± 6.6^{c}	114.6 ± 0.6^{a}	142.0 ± 5.8^{a}	219.6 ± 4.1^{a}
OVA+OP	**OVA**	28.9 ± 2.1^{c}	183.3 ± 9.5^{b}	451.9 ± 11.6^{a}	24.8 ± 0.6^{b}	65.6 ± 3.4^{b}	243.7 ± 16.2^{a}
OVA+OA		49.1 ± 2.4^{b}	113.5 ± 1.4^{c}	124.3 ± 3.4^{b}	18.9 ± 3.5^{b}	24.0 ± 1.8^{c}	208.1 ± 19.0^{a}
LYS		133.6 ± 9.5^{a}	493.3 ± 15.3^{a}	83.0 ± 8.1^{b}	39 ± 2.4^{b}	92.3 ± 5.0^{b}	106.9 ± 26.4^{a}
LYS+LP	**LYS**	165.3 ± 7.4^{a}	389.9 ± 18.4^{b}	162.7 ± 8.6^{a}	54.5 ± 2.8^{a}	121.1 ± 6.5^{a}	120.4 ± 12.3^{a}
LYS+LA		161.5 ± 11.1^{a}	352.7 ± 15.1^{b}	151.0 ± 6.9^{a}	35.9 ± 2.4^{b}	109.1 ± 2.5^{ab}	114.2 ± 7.9^{a}
OM		41.2 ± 0.3^{a}	59.0 ± 6.7^{a}	92.1 ± 5.5^{a}	23.2 ± 0.85^{b}	37.8 ± 1.9^{b}	14.3 ± 4.0^{b}
OM+MP	**OM**	69.0 ± 12.1^{a}	83.9 ± 2.3^{a}	91.2 ± 8.2^{a}	62.6 ± 11.3^{a}	131.0 ± 8.8^{a}	92.6 ± 7.5^{a}
OM+MA		54.5 ± 11.5^{a}	83.7 ± 8.1^{a}	79.8 ± 2.5^{a}	16.0 ± 1.2^{b}	33.7 ± 2.3^{b}	15.8 ± 0.8^{b}

However, the hydrolysates of LYS only modestly reduced the secretion of IL-5 stimulated by LYS in splenocytes from mice mono-sensitized to this protein. The hydrolysates of OM failed to impair the production of these cytokines, which even increased when the hydrolysate of OM with pepsin was present in the culture ($P < 0.05$), in the case of the poly-

sensitized animals. On the other hand, the hydrolysates of OVA and LYS with both enzymes tended to promote the release of the Th1 cytokine TNF-α, when used together with the intact proteins for stimulation of the spleen cells from mono-sensitized mice (Table 4.3.3).

In spleen cells from mice sensitized to EW, the hydrolysates of OVA, unlike those of LYS and OM, significantly reduced the EW-induced secretion of both IL-4 and IL-5 (the hydrolysate of LYS with pepsin also significantly decreased the release of IL-4). In addition, the hydrolysates of OVA and LYS with pepsin increased the production of TNF-α (Table 4.3.4). These results thus indicate that the hydrolysate of OVA with pepsin was able to bias allergen-specific immune responses, helping to restore the Th1/Th2 balance.

Table 4.3.4. Immunomodulating effects of the hydrolysates of OVA (O), LYS (L) and OM (M) with pepsin (P) and alcalase (A). Splenocytes from EW-sensitized mice were stimulated with EW (200 μg/mL) in the absence or presence of the hydrolysates (200 μg/mL), and the secretion of IL-4, IL-5, and TNF-α was evaluated. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$) for each cytokine.

	EW-sensitized mice		
	IL-4	IL-5	TNF-α
EW	127.9 ± 13.0^{a}	161.7 ± 6.0^{a}	118.1 ± 10.3^{bcd}
EW+OP	59.1 ± 11.5^{bc}	67.6 ± 13.8^{c}	157.7 ± 6.9^{a}
EW+OA	35.6 ± 11.0^{c}	18.1 ± 3.4^{d}	107.9 ± 15.8^{cd}
EW+LP	104.7 ± 8.3^{a}	123.2 ± 7.8^{b}	148.6 ± 8.9^{ab}
EW+LA	94.8 ± 6.8^{ab}	162.6 ± 2.6^{a}	124.7 ± 8.3^{a}
EW+MP	115.9 ± 10.3^{a}	130.6 ± 12.3^{ab}	96.4 ± 2.2^{dc}
EW+LA	109.3 ± 4.2^{a}	135.5 ± 1.5^{ab}	83.4 ± 6.6^{d}

4.3.1.4. Chromatographically enriched peptide fractions exert distinct Th2 and Th1 cytokine-producing effects

With the aim to enrich and identify bioactive peptide sequences, the hydrolysate of OVA with pepsin (OP), which had shown the most promising properties in terms of reduction of Th2 immunity and enhancement of Th1 responses, was separated by RP-HPLC at a semi preparative scale into 5 fractions (labelled RP-OP1 to RP-OP5) and by anion exchange FPLC in

2 fractions (AE-OP1 and AE-OP2). These were collected after about 30 runs to obtain enough material to determine protein concentration and immunostimulating and immunomodulating activities on splenocytes from EW-sensitized mice, before being analysed by RP-HPLC-MS/MS.

In the hydrolysate of OVA with pepsin, RP-HPLC fractions RP-OP1 and RP-OP2 stimulated the lowest production of the Th2 cytokines IL-4 and IL-5, and RP-OP3, RP-OP4 and RP-OP5, the highest. Fraction RP-OP2 considerably reduced the production of both IL-4 and IL-5 induced by EW in the splenocytes, while RP-OP4 enhanced the production of IL-4 and did not modify that or IL-5 (Fig. 4.3.6a and c). Spleen cells from EW-sensitized mice responded to stimulation with fractions RP-OP3, RP-OP4 and RP-OP5 releasing IL-12p70. IL-12 favours DC-driven differentiation of CD4+ T cells to IFN-γ-producing Th1 cells when it is present at an early stage of T cell activation (Macatonia et al., 1995). Furthermore, when added together with EW, RP-OP4 and RP-OP5, as well as OP, enhanced the release of IL-12p70 (Fig. 4.3.6b). However, the whole hydrolysate promoted IFN-γ secretion induced by EW to a much higher extent than any of the individual RP-HPLC fractions (Fig. 4.3.6d). On the other hand, the first fraction obtained by anion exchange chromatography (AE-OP1) induced weaker Th2 (IL-4 and IL-5) and Th1 responses (IFN-γ), compared with the second fraction (AE-OP2), although it significantly enhanced IFN- γ production by EW (Fig. 4.3.6).

The peptides present in the hydrolysate of OVA with pepsin and its isolated fractions were identified by mass spectrometry (Annexes I-VIII). In the RP-HPLC fractions, peptides ranging from 5 to 22 aminoacids were found, with some coincident sequences, mainly between contiguous fractions. In turn, in AE-OP1 and AE-OP2, we identified, respectively, 67 and 64 peptides distributed along the whole OVA molecule, with 16 identical sequences, although they shared many analogous peptides with amino- and carboxy-terminal truncations. Fig. 4.3.7 shows the fragments identified in the most potent immunomodulatory fractions: RP-OP2, RP-OP4, AE-OP1 and AE-OP2 (their respective RP-HPLC chromatograms are shown in Figs. 4.3.8 and 4.3.9). The comparison between RP-OP2 and RP-OP4, which exerted distinct Th2 and Th1

cytokine-producing effects in combination with EW, showed a different occurrence of sequences within wide parts of the protein. In particular, RP-OP4 lacked peptides in the areas 98-227, 248-275, 285-360 and 366-386. Interestingly, fractions RP-OP2 and AE-OP1, that down-regulated Th2 responses, shared 26 peptides in common, some of which, such as those within the areas 205-211 and 369-376, were absent from both RP-OP4 and AE-OP2, which exerted the opposite effect. However, the complex peptide composition of these fractions hindered the identification of sequences that could play a role to diminish Th2 responses and, in general, the correlation of peptide structure and immunoregulating function.

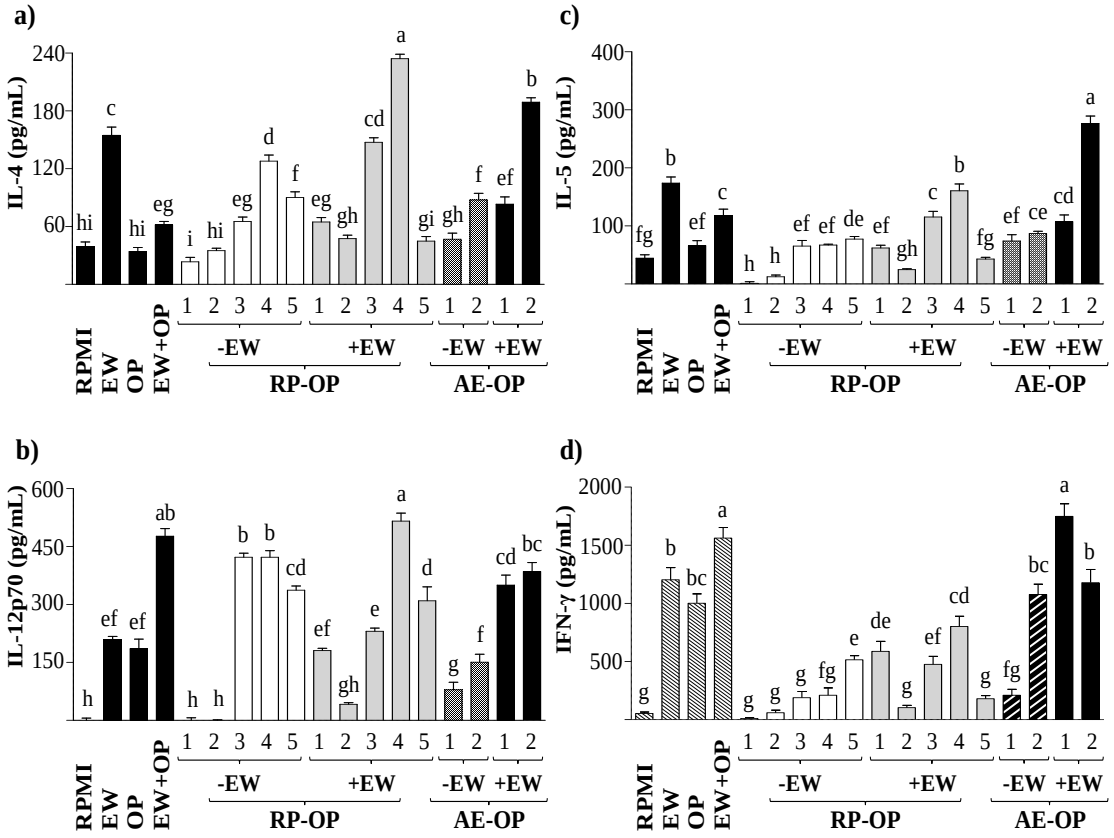

Figure 4.3.6. Immunostimulating and immunomodulating effects of the fractions obtained from the hydrolysate of OVA with pepsin (OP) by semi-preparative RP-HPLC (RP-OP1 to RP-OP5) and anion exchange chromatography (AE-OP1 and AE-OP2). Splenocytes from EW-sensitized mice were stimulated with OP and its fractions (200 μg/mL) in the absence or presence of EW (200 μg/mL), and the secretion of IL-4 (a), IL-5 (b), IL-12p70 (c) and IFN-γ (d) was evaluated. Data are expressed as means ± SEM. Different letters indicate statistically significant differences ($P < 0.05$).

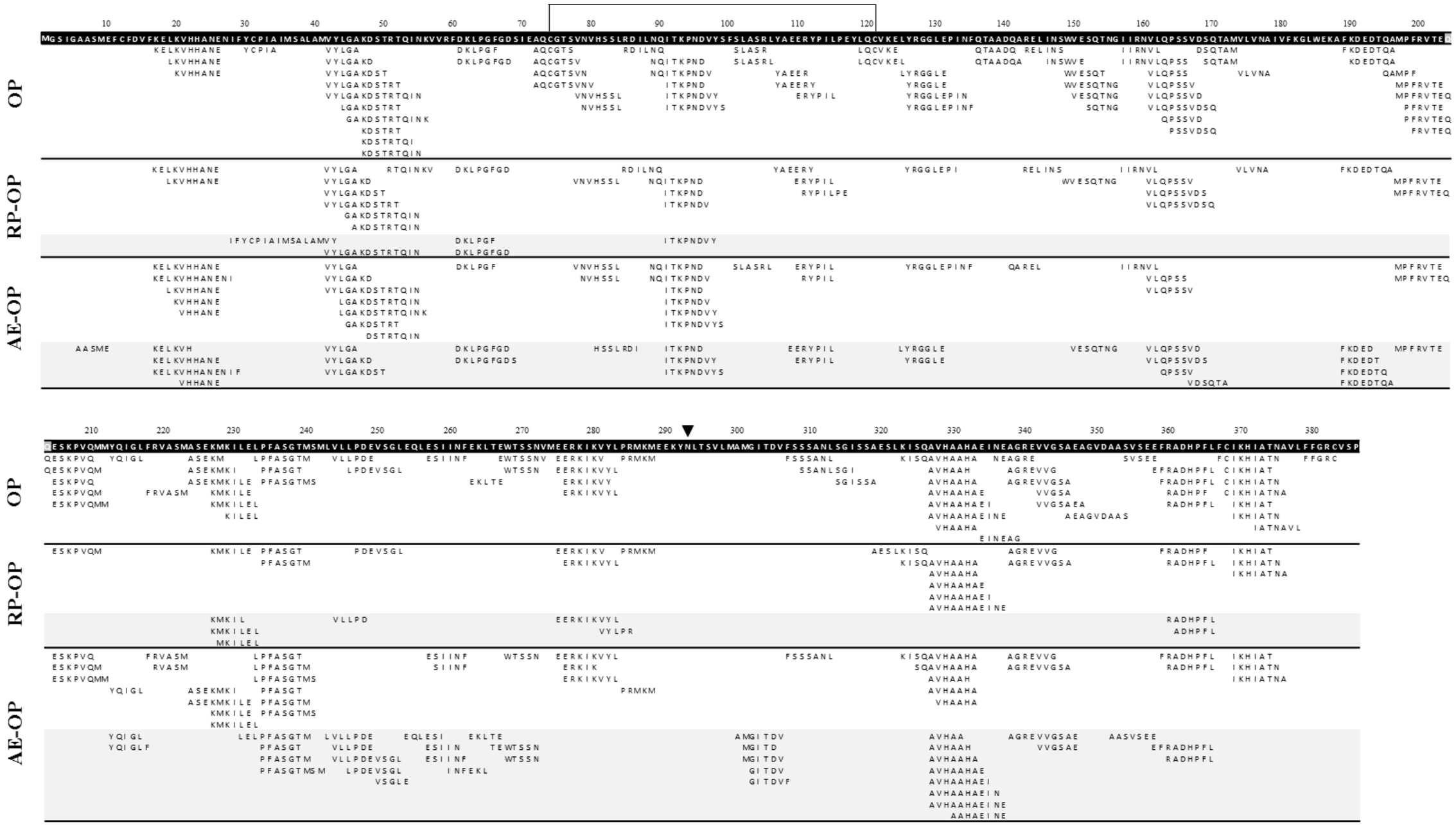

Figure 4.3.7. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the hydrolysate of OVA with pepsin (OP), and in the fractions separated by semi-preparative RP-HPLC: RP-OP2 (white ☐) and RP-OP4 (light grey ☐) and anion exchange chromatography: AE-OP1 (white ☐) and AE-OP2 (light grey ☐). ▼: Carbohydrate chains. ⌜⌝: Disulphide bonds.

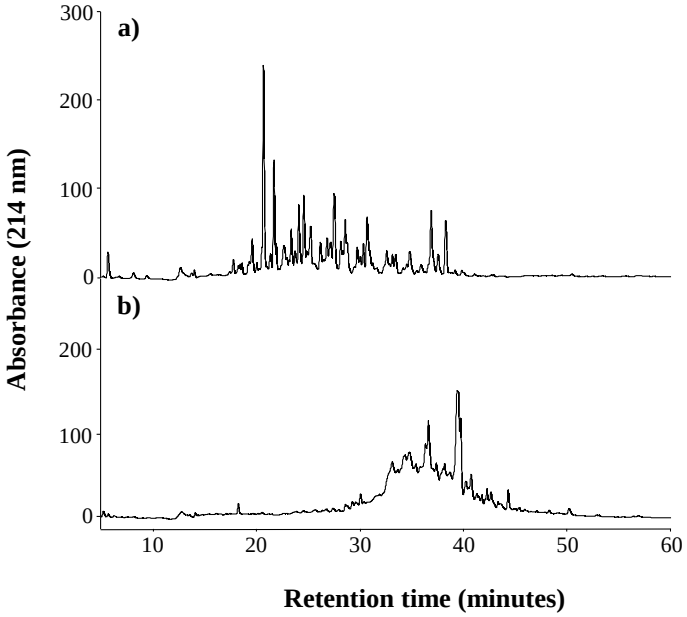

Figure 4.3.8. RP-HPLC patterns of the peptide fractions obtained from the hydrolysate of OVA with pepsin (OP) by semi-preparative RP-HPLC: RP-OP2 (a) and RP-OP4 (b).

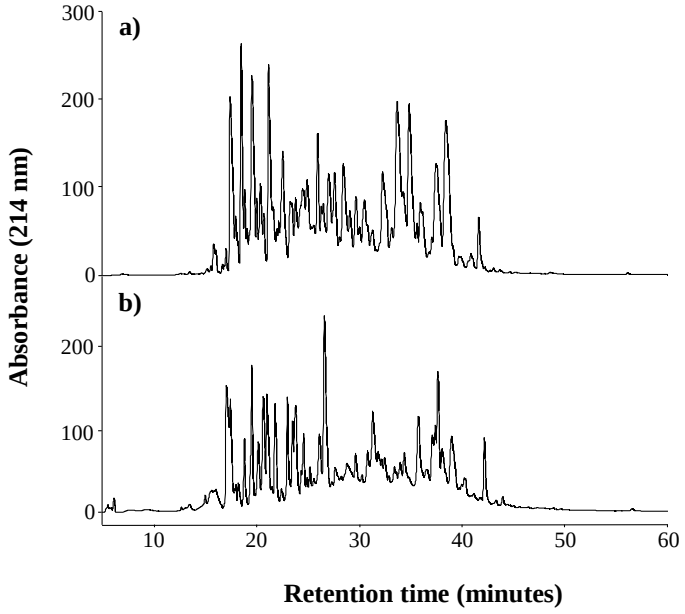

Figure 4.3.9. RP-HPLC patterns of the peptide fractions obtained from the hydrolysate of OVA with pepsin (OP) by anion exchange chromatography: AE-OP1 (a) and AE-OP2 (b).

4.3.2. Discussion

This study evidences the immunoregulatory role of OVA hydrolysed with pepsin and alcalase on splenocytes from mice orally sensitized to OVA, as well as on splenocytes from mice orally sensitized to identical amounts of OVA, OM and LYS. In both models, OVA

hydrolysates induced a low Th2 activation and significantly suppressed the release of IL-4 and IL-5 promoted by intact OVA, stimulating the secretion of the Th1 cytokine TNF-α. Furthermore, OVA hydrolysates combined the capacity to modulate the immune response *ex vivo* with a reduced ability to trigger cutaneous allergic symptoms *in vivo* in a PCA assay.

Interestingly, the hydrolysates of OVA, also significantly inhibited the EW-induced secretion of IL-4 and IL-5 in mice sensitized to EW, which consists of 54% OVA, 11% OM and 3.4% w/w LYS. This observation suggests that the sole administration of OVA hydrolysates could result in significant protection against bystander allergens. In fact, immunotherapy with OM-depleted heated EW effectively protects from allergic reactions to BALB/c mice sensitized to EW, despite OM is being considered an immunodominant allergen in this mouse model (Jiménez-Saiz et al., 2011).

The results reported in section 4.2 showed that the hydrolysates of egg white proteins with alcalase down-regulated the production of Th2 cytokines and the secretion of IgE by human PBMCs from non-allergic donors artificially Th2-skewed by culture with IL-4 and anti-CD40; while, in the same cell model, hydrolysates with pepsin stimulated the restoration of the Th1/Th2 balance by the simultaneous decrease in IL-5 and IL-13 and increase in IFN-γ. The present results basically confirm those findings, in accordance with Holvoet et al. (2013), who reported parallel outcomes with the non-antigen-specific model of Th2-skewed human PBMCs and allergen-primed murine splenocytes. However, the assay using spleen cells from egg white protein-sensitized mice provided a more refined characterization of the cytokine responses produced by the hydrolysates from OVA, LYS and OM, in line with the proven suitability of the BALB/c mouse model to stablish mechanisms and preventive or therapeutic strategies against food allergy and the fact that the spleen is a source of both antigen-presenting cells and allergen-specific T cells (Yang and Mine, 2009; Pablos-Tanarro et al., 2016). Moreover, the use of splenocytes from sensitized BALB/c mice enabled the assessment of the ability of the hydrolysates to modulate the specific T cell responses induced by the sensitizing allergens, which allowed discriminating OVA hydrolysates as the most promising therapeutic compounds.

The observation that complete hydrolysis of OVA and LYS with pepsin reduced their ability to promote Th2 effector pathways highlights the importance that resistance to pepsin digestion plays in the allergenicity of these proteins (Benedé et al., 2015). In contrast, both intact OM and its hydrolysate with pepsin induced very similar cytokine profiles. OM (whose structure is stabilized by 9 disulphide bridges) is proteolysed by pepsin in minutes, but the resulting peptides form big fragments linked by disulphide bonds (Benedé et al., 2013). OM, therefore, behaves as certain food allergens that, despite being digested, retain the T cell stimulatory properties of the intact protein, such as Ara h 1 from peanut (Eiwegger et al., 2006). This fact disagrees with the outcomes of the experiments with Th2-skewed PBMCs (section 4.2), in which case the hydrolysate of OM with pepsin down-regulated the production of Th2 cytokines, pointing up the enhanced capacity of the model used in this section to discriminate more accurately among hydrolysates with immunomodulating potential.

The hydrolysate of OVA with alcalase, unlike that of OVA with pepsin, exerted on spleen cells from sensitized mice a negligible Th2-stimulating role. A likely explanation is that all the hydrolysates with alcalase contains short peptides, with molecular masses lower than 1.5 kDa, that provide a poor sequence coverage (sections 4.1 and 4.2). This possibly correlates with a low proportion of full length T epitopes with the minimum core sequence of 9 amino acids required for recognition by reactive T cells (Prickett et al., 2011). For its part, the hydrolysate of OVA with pepsin could be regarded as a good candidate for peptide-based immunotherapy on the grounds of its potential to reduce Th2 responses induced by egg allergens, but also to enhance Th1 responses. In fact, the peptides contained in this hydrolysate increased the production of DC-derived cytokines, such as IL-12 which, in turn, translated into an enhanced capacity to induce Th1 cell activation, as evidenced from the IFN-γ secretion in the spleen cell culture.

Semi-preparative chromatographic separation of OVA hydrolysed with pepsin yielded peptide fractions with different immunostimulating and immunomodulating properties. In particular, hydrophilic peptides (RP-OP2) and peptides with low affinity for the anion exchange

column (AE-OP1) hindered the secretion of Th2 cytokines stimulated by EW in the spleen cells from EW-sensitized mice (in contrast to RP-OP4 and AE-OP2, respectively). Cationic host-defense peptides are known to exert immunomodulating properties beyond their direct antimicrobial action through the stimulation of innate immune responses and the modification of T and B cell responses (Hilchie et al., 2013). Similarly, cationic peptides, from bovine β-lactoglobulin, unlike anionic ones, are tolerogenic and prevent β-lactoglobulin sensitization in mice (Pecquet et al., 2000). However, despite there were similarities in the peptides contained in RP-OP2 and AE-OP1, with common sequences absent from other fractions, it was not possible to trace peptide composition to Th2 down-regulating activities. Furthermore, the observation that peptide enrichment by reverse-phase and anion-exchange fractionation of the hydrolysate did not lead to an increase in its immunomodulating efficiency suggests that factors other than peptide hydrophilicity or charge influence the ability to bias an allergic immune response. In fact, so far, very little is known regarding the structural and sequence requirements underlying the immunoregulatory properties of peptides (Haney and Hancock, 2013).

It is noteworthy that the whole hydrolysate of OVA with pepsin, as well as all its derived fractions, except for RP-OP4, contained several peptides included in the immunodominant sequence OVA (323-339), which comprises multiple T cell epitopes restricted by the MHC class I-Ad molecule of BALB/c mice, such as OVA (324-332) (SQAVHAAHA), OVA (327-335) (VHAAHAEIN) and OVA (329-337) (AAHAEINEA) (Robertson et al., 2000). Furthermore, the hydrolysate and certain fractions contained peptides related to OVA (39-50), OVA (147-158) and OVA (263-274), which, as well as OVA (329-340), induce proliferation and IFN-γ secretion in spleen cell of BALB/c mice subcutaneously sensitized to OVA, without stimulating the production of IL-4 (Yang and Mine, 2009). The occurrence in the whole OVA hydrolysate of a combination of immunogenic structures with synergistic or adjuvant actions could explain its stronger immunomodulating potential as compared with its separated peptide fractions (Yang et al., 2009b and 2010; Bøgh et al., 2012).

The assessment of the immunostimulating and immunomodulating capacity of hydrolysates of egg white proteins on splenocytes from sensitized BALB/c mice showed that the hydrolysate of OVA with pepsin stimulated low Th2 responses and also down-regulated Th2 responses induced by intact egg allergens *ex vivo*, while contributing to increase Th1 cytokine secretion. Hydrolysis of OVA with pepsin appears as a cheap, simple and safe approach for a reproducible production of immunomodulating peptides with a reduced ability to trigger allergic symptoms. Altogether, these results encouraged us to validate *in vivo* the potential immunomodulating effects of this hydrolysate as a treatment strategy of egg allergy.

4.4. *In vivo* evaluation of the sensitizing capacity, preventive ability and therapeutic effects of hydrolysed ovalbumin

The previous sections (4.1, 4.2 and 4.3) demonstrated that the hydrolysates of OVA with pepsin and alcalase were hypoallergenic *in vitro* and *in vivo* (section 4.2 and 4.3). In addition, these presented immunomodulating effects not only on unspecific cell models (section 4.1 and 4.2), but also on a more accurate *ex vivo* model based on cells from mice sensitized to egg allergens (section 4.3). In general, OVA hydrolysed with pepsin and alcalase has shown a reduced ability to induce Th2 responses and a marked capacity to decrease the allergic immune responses induced by the egg allergens.

The use of immunomodulating peptides and hydrolysates has previously been suggested to avoid the development of allergic symptoms in sensitized individuals or even, as a prior strategy, to prevent sensitization to intact allergens (Adel-Patient et al., 2011; Hacini-Rachinel et al., 2014; Valenta et al., 2016). However, the preventive usage of allergen derived hydrolysates poses a risk in the development of the sensitization against the preparation itself, being necessary to assure that these hydrolysates lack sensitizing potential (van Esch et al., 2013). On the other hand, the therapeutic administration of immunomodulating peptides represent a safer alternative to full allergens, as they produce fewer side effects and increase adherence to therapy (Yang et al., 2010; Casale and Stokes, 2011; Moldaver and Larché, 2011).

Given this scenario, the last step of this thesis was the *in vivo* evaluation of the sensitizing capacity, preventive ability and therapeutic effects of OVA hydrolysed with pepsin and alcalase in a murine model. For this propose, BALB/c mice were orally administered the hydrolysates and their ability to induce the production of specific antibodies, as well as to elicit anaphylactic responses, were evaluated. We then studied their potential preventive and therapeutic ability in mice sensitized to EW before or after the administration of the hydrolysates, respectively, compared with those of the intact OVA. Finally, in order to understand the immunological events underlying the sensitization potential, the tolerance induction after pre-treatment and the desensitization after therapeutic administration, we evaluated the changes in cell populations at systemic levels and also studied the variations on relative gene expression at intestinal level.

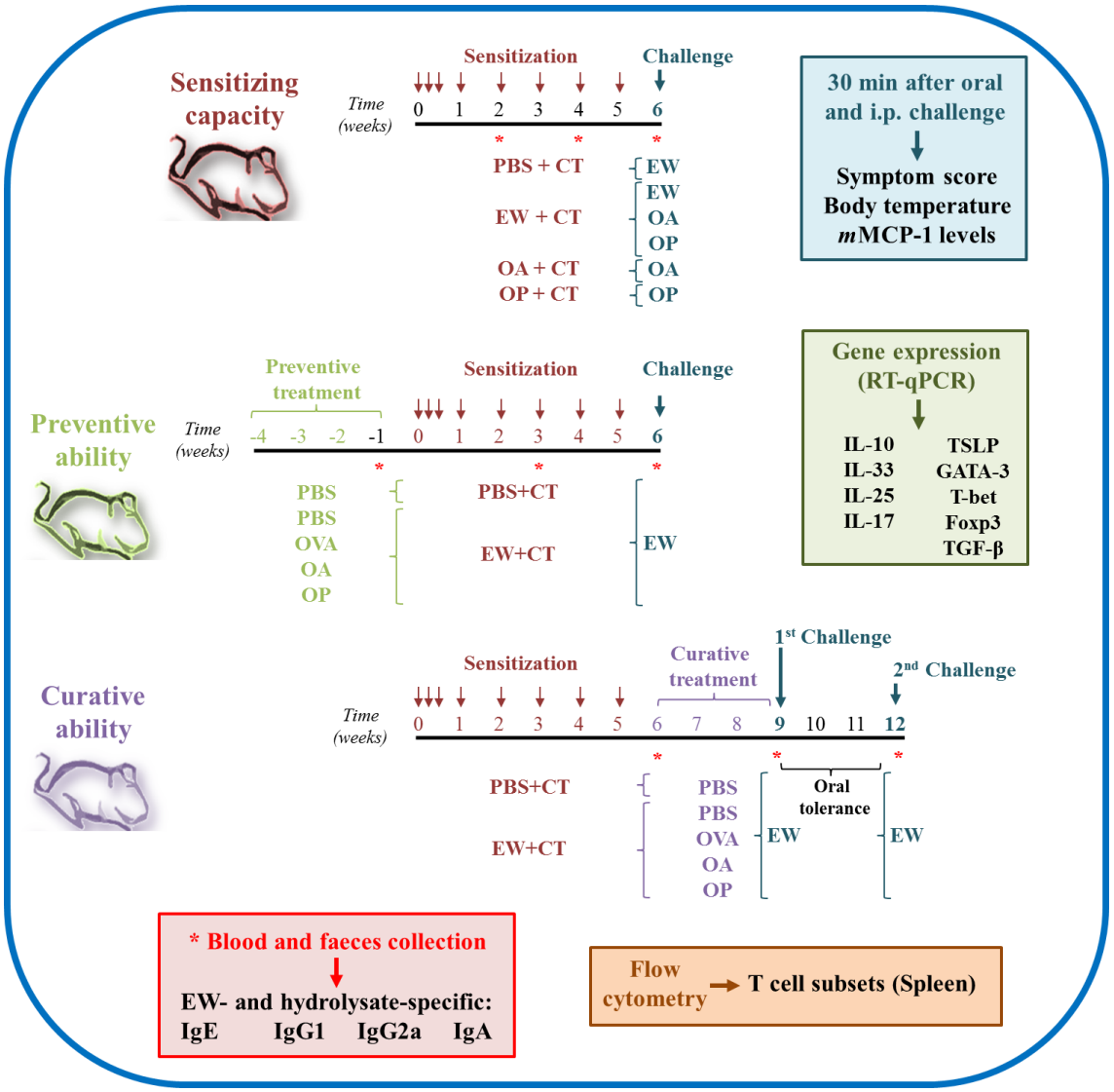

Figure 4.4.1. Scheme of the experimental procedure followed in section 4.4. EW, egg white; Foxp3, forkhead box protein P3; GATA-3, trans-acting T-cell-specific transcription factor; Ig, immunoglobulin; IL, interleukin; mMCP-1, mouse mast cell protease I; OA, OVA hydrolysed with alcalase; OP, OVA hydrolysed with pepsin; OVA, ovalbumin; T-bet, T-box transcription factor; TSLP, thymic stromal lymphopoietin.

4.4.1. Results

4.4.1.1. OVA hydrolysed with pepsin lack sensitizing and eliciting capacity, but its hydrolysis with alcalase revealed immunogenic and antigenic effects

EW-specific IgE, IgG1, IgG2a and IgA were only detected in mice administered EW plus CT -EW+CT- (Fig. 4.4.2), thus showing that the hydrolysates did not hold sensitizing potential to the intact protein from which they derived, that constitutes 54% (w/w) of EW. While the hydrolysates were not able to sensitize to OVA, OA plus CT -OA+CT- induced the production of OA-specific antibodies, although to a much lower level than the EW-specific antibodies generated by EW+CT (Fig. 4.4.3a-d). Administration of OP plus CT -OP+CT- gave rise to low, but detectable OP-specific IgG2a, but not to specific IgE, IgG1 or IgA responses, which is indicative of a low immunogenicity (Fig. 4.4.3e and f). IgE, IgG1, IgG2a and IgA antibodies present in serum from mice sensitized to EW cross-reacted to OA, whereas IgG1 and IgG2a antibodies cross-reacted to OP, suggesting that some of the peptides contained in the hydrolysates carried epitopes able to bind to the antibodies induced by the administration of EW+CT. Mice administered OA+CT, unlike those administered OP+CT, underwent clinical signs and temperature drops following oral challenge with the hydrolysate used for sensitization. Anaphylactic symptoms were equivalent to those experienced by mice sensitized to EW+CT and orally challenged with EW, as it was rectal temperature (Fig. 4.4.4a and b). This points out that OA held sensitizing potential, although the induction of specific antibodies did not correlate with the ability of the hydrolysate to provoke anaphylaxis. However, subsequent i.p. challenge did not increase the severity of symptoms in mice administered OA+CT, leading to lower scores and rectal temperature values than the previous oral challenge, as well as to negligible serum levels of mMCP-1 (Fig. 4.4.4c and d).

The assessment of the allergenic potential of the hydrolysates in mice sensitized to EW revealed that oral and i.p. challenges with OA elicited significant clinical signs, accompanied by temperature drops and release of serum mMCP-1 (Fig. 4.4.4e). This shows that OA was not

hypoallergenic, even if OA-induced anaphylaxis was, in general, lower than that provoked by EW. Unlike OA, OP did not stimulate allergic reactions in EW+CT administered mice, as judged by clinical sings, rectal temperature values and serum mMCP-1 levels.

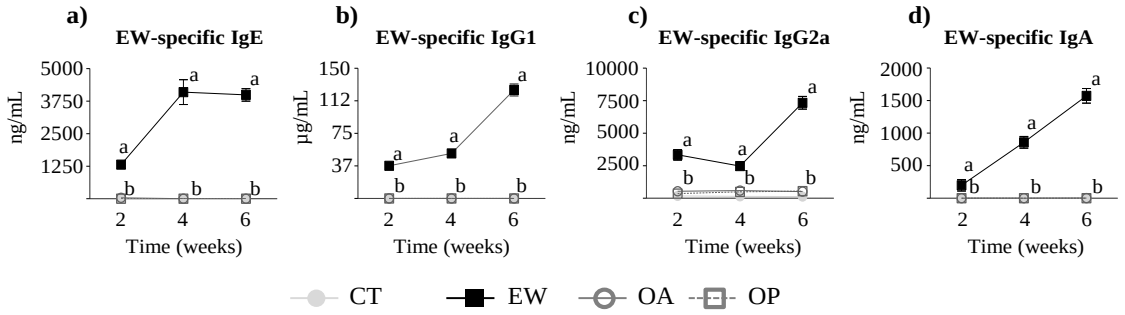

Figure 4.4.2. Specific antibody responses induced against EW during administration of CT or, EW, OA and OP plus CT. Levels of EW-specific IgE (a), IgG1 (b), IgG2a (c) and IgA (d) were determined by ELISA in serum samples from CT (○), EW (■), OA (○) and OP (□) groups collected on weeks 2, 4 and 6 of the sensitization period. Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$) between the groups of mice for the same experimental point.

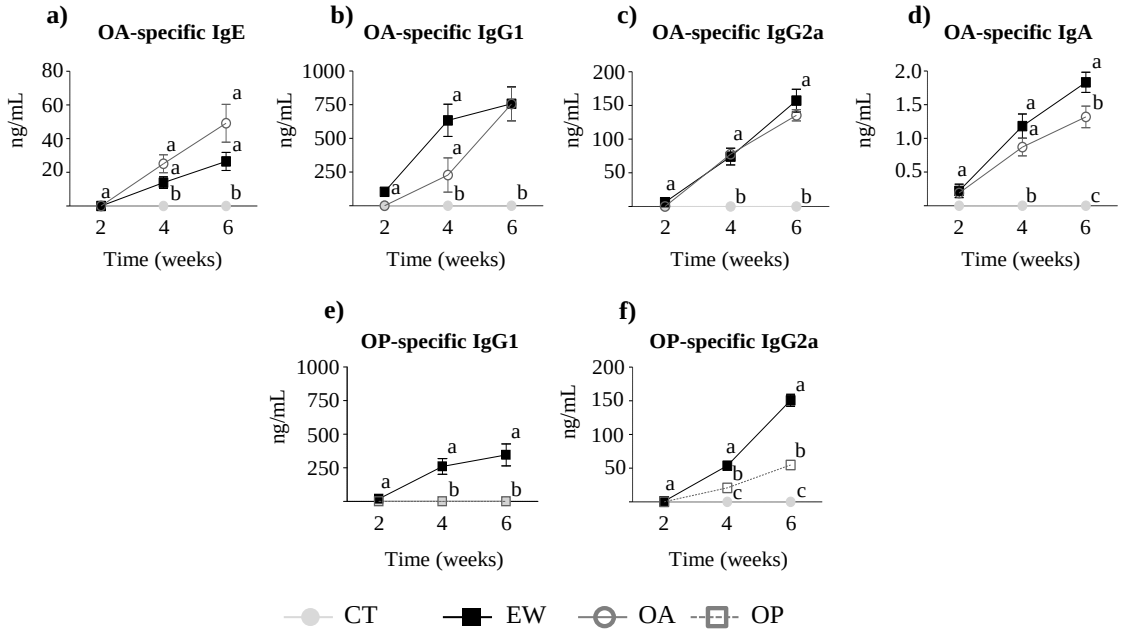

Figure 4.4.3. Specific antibody responses induced against the hydrolysates, OA and OP, during administration of CT or, EW, OA and OP plus CT. Levels of specific IgE (a), IgG1 (b), IgG2a (c) and IgA (d) against OA, and specific IgG1 (e) and IgG2a (f) against OP, were determined by ELISA in serum samples from CT (○), EW (■), OA (○) and OP (□) groups collected on weeks 2, 4 and 6 of the sensitization period. Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$) between the groups for the same experimental point.

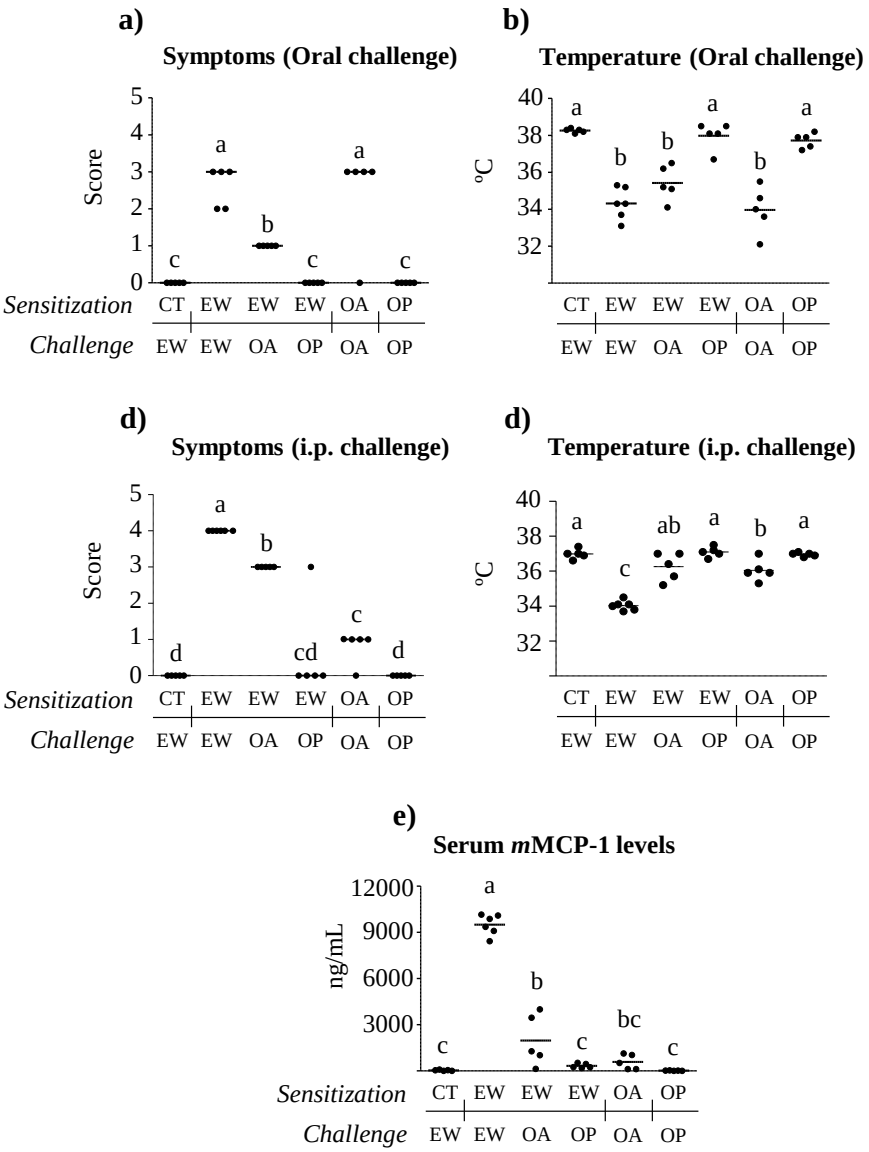

Figure 4.4.4. Systemic anaphylactic responses after oral (a,b) and i.p. (c,d,e) challenge. Sham- (CT) and EW-sensitized mice were challenged with EW, while mice administered OA and OP plus CT were challenged with the corresponding hydrolysate. Eliciting capacity was determined by challenge of EW-sensitized mice with OA and OP. Anaphylactic score (a,c) and drop in body temperature (b,d) were determined 30 minutes after each challenge. Release of mMCP-1 (e) was quantified in serum samples collected after sacrifice (week 6). Data are represented individually for each mouse. Clinical scores are expressed as medians and, body temperature and levels of mMCP-1 expressed as means (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

We next decided to evaluate the binding preferences of the antibodies raised in mice by the administration of EW+CT and OA+CT. To this aim, we first conducted PCA assays to assess the participation of IgE and IgG1 in the development of allergic responses to EW and OA. Heat-treatment of the sera from EW+CT and OA+CT administered mice at 56°C for 3 h,

before their intradermal administration to naïve mice, significantly reduced the extent of dye extravasation following intravenous challenge with the protein used for sensitization, although it did not eliminate it (Fig. 4.4.5). This suggests that, in addition to the more heat-sensitive IgE antibodies, IgG1 antibodies also contributed to anaphylaxis in both cases. Subsequently, considering the potential role of IgG1 in allergic manifestations and the fact that the higher levels of IgG1 antibodies aided their quantification, we conducted inhibition ELISA experiments to examine the binding capacity of IgG1 from EW+CT and OA+CT sensitized mice (Fig. 4.4.6).

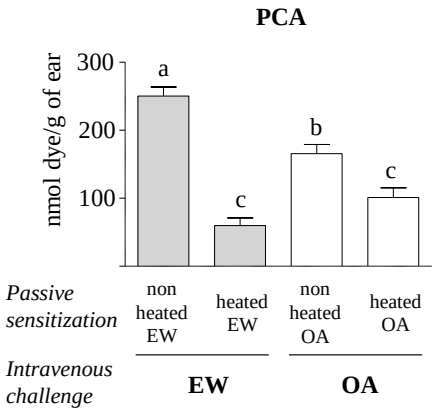

Figure 4.4.5. PCA assay with unheated and heated (56°C for 3 h) pooled sera from mice administered EW or OA plus CT. Allergen-specific dye extravasation was calculated by correcting the EW- or OA-induced absorbance with the absorbance of the supernatant of the ear injected with pooled control sera. Data are means ± SEM (n= 2). Different letters indicate statistically significant differences ($P < 0.05$).

IgG1 of sera from EW+CT administered mice had low affinity for OP and lower affinity for OA than for EW. Furthermore, these antibodies bound with very low strength the fraction of OA with molecular mass below 10 kDa, raising the question as to whether the allergic response induced by this hydrolysate on EW sensitized mice was nonspecific, but due to an antigenic substance belonging to the enzymatic preparation (Fig. 4.4.6a). In fact, IgG1 present in sera from OA+CT sensitized mice bound with lower affinity the fraction of the hydrolysate with molecular mass below 10 kDa, comprising the fragments from OVA hydrolysis, than the corresponding fraction with molecular mass above 10 kDa, which contained

the enzyme, or the inactivated alcalase preparation itself (Fig. 4.4.6b). These observations suggest that the enzyme preparation was immunogenic and highly antigenic.

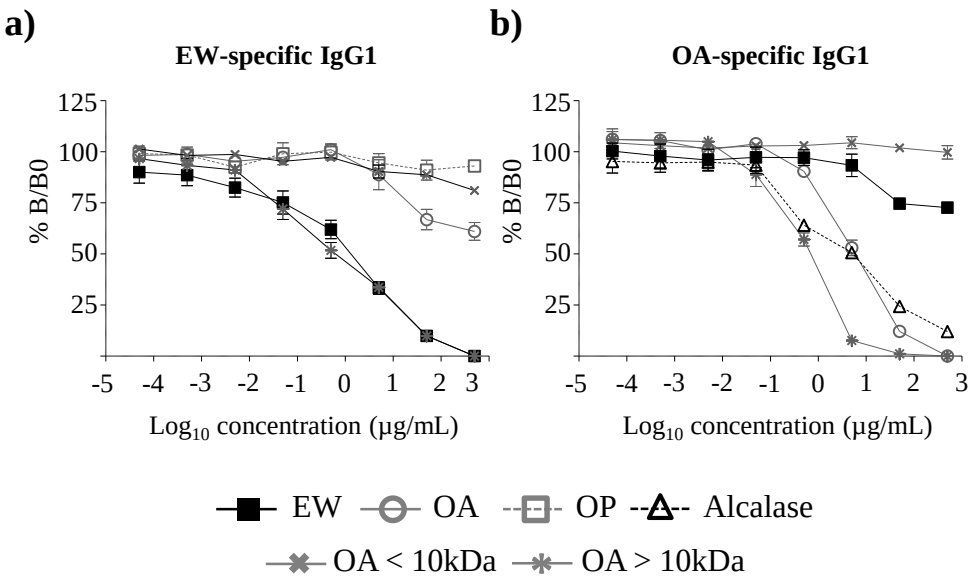

Figure 4.4.6. IgG1-binding capacity using sera from mice administered EW (a) or OA (b) plus CT. Comparison of the inhibitory capacity was assessed by pre-incubation of pooled sera (n=5) obtained at sacrifice (week 6) with tenfold dilutions (0.05 pg/mL-500 µg/mL) of inhibitors: EW (■), OA (○), OP (□), OA above 10 kDa (*), OA below 10kDa (✖) and the enzymatic extract of alcalase (▲). IgG1 inhibition curves show data expressed as means ± SEM of three independent assays.

The expression levels of the intestinal Th2-skewing cytokines IL-33, IL-25 and TSLP, as well as the transcription factors GATA-3, T-bet and Foxp3 which specify, respectively, the development of Th2, Th1 and T reg cells, was assessed in the duodenum 3 h after the i.p. challenge (Fig. 4.4.7). A significant upregulation of IL-33 was detected in mice administered EW, OA and OP plus CT, and challenged with the respective protein preparation, as compared with sham-sensitized mice, whereas IL-25 and TSLP were only upregulated in mice sensitized to EW. While it is likely that intestinal injury resulting from mast cell activation due to the oral challenge, and not just the sensitization status, influenced the gene regulation of these cytokines at the duodenal level, as judged by the different IL-33, IL-25 and TSLP responses induced by EW, OA and OP on EW-sensitized mice, these results suggest that challenge with the protein preparation used for previous administration with CT boosted intestinal IL-33 and GATA-3

expression, even in the absence of detectable intestinal or systemic symptoms (Fig. 4.4.7a and d). Meanwhile, administration of OA+CT and OP+CT did not change the expression level of T-bet or Foxp3 in the duodenum with respect to sham-sensitized mice (Fig. 4.4.7e and f).

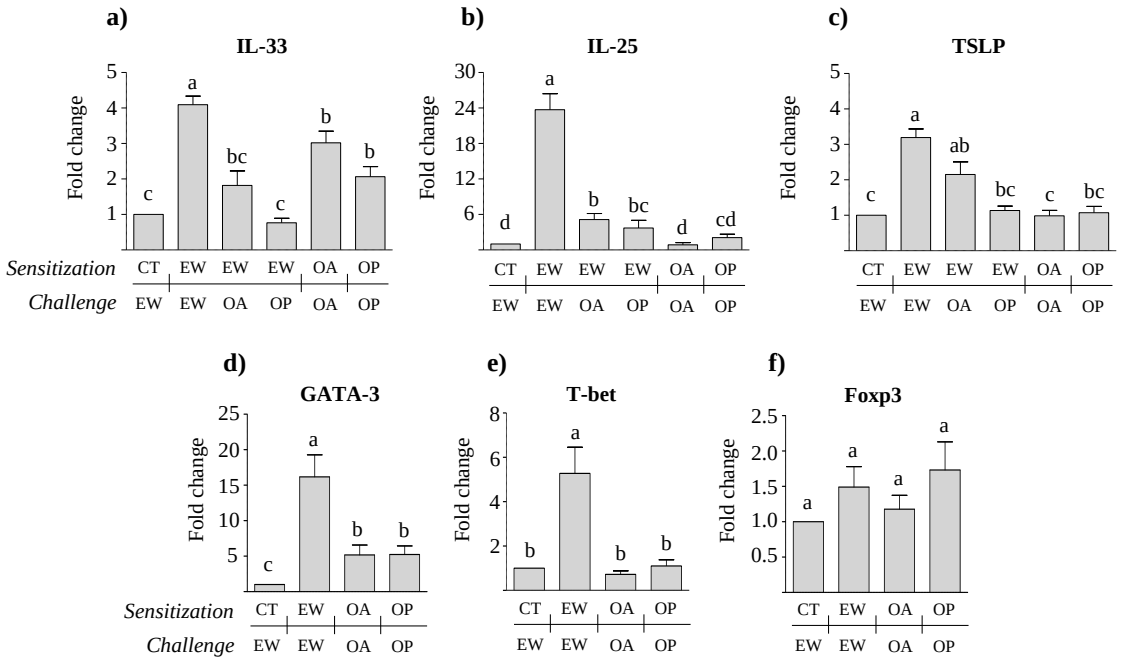

Figure 4.4.7. Intestinal expression, determined by RT-qPCR, of IL-33 (a), IL-25 (b), TSLP (c), GATA-3 (d), T-bet (e) and Foxp3 (f) in duodenum samples of mice administered EW, OA or OP plus CT. Results were normalized to β-actin and expressed relative to sham-sensitized group (identified as CT). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$).

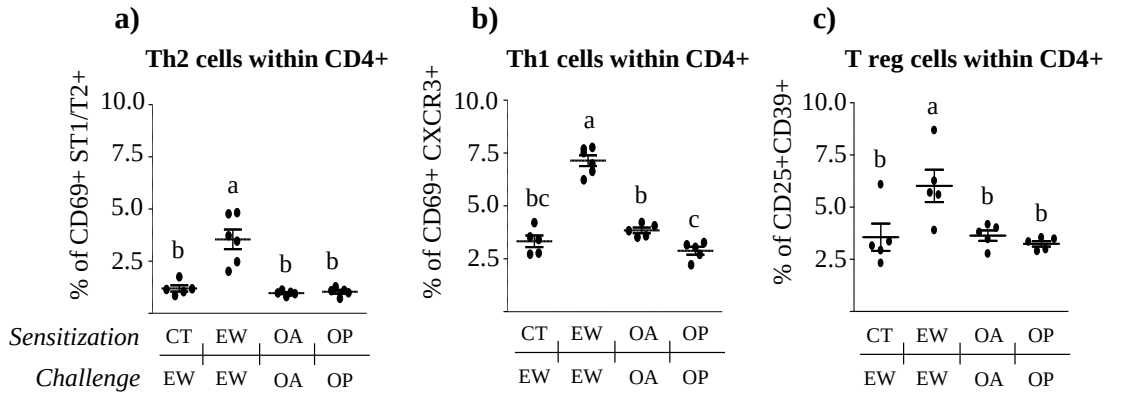

Figure 4.4.8. The percentages of activated Th2 (a), Th1 (b) and T reg (c) cells within the CD4+ population in spleens from mice administered CT or, EW, OA and OP plus CT determined by flow cytometry. Individual values are represented for each mice and data expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

In parallel with the regulation of the transcription factors GATA-3, T-bet and Foxp3 in the intestine, an increased percentage of activated Th2 and Th1 cells, and no change in T reg cells, was found in the spleen of mice administered EW+CT. Accordingly, the proportion of activated Th2, Th1 and T reg cells were comparable in mice administered OA and OP plus CT and in sham-sensitized mice (Fig. 4.4.8). When the specific response of splenocytes from mice administered EW, OA and OP plus CT was studied by re-stimulation with the protein preparation used for sensitization, the highest release of Th2 (IL-4 and IL-5), Th1 (IFN-γ) and regulatory (IL-10) cytokines was found in mice administered EW+CT, while no significant production was detected from the spleen cells of mice administered OP+CT (Fig. 4.4.9). The spleen cells from mice administered the hydrolysates plus CT did not respond to EW stimulation, except for a significant release of IL-17. On the other hand, mice sensitized to EW+CT responded to re-stimulation with OA with cytokine levels similar to those induced by the negative control (RPMI) and to re-stimulation with OP with a lower release of IL-4 and IL-5 than that induced by EW, but with equivalent levels of IFN-γ (Fig. 4.4.9). This is in agreement with previous results that suggested that OP was able to restore a biased Th2/Th1 balance on spleen cells of OVA- and EW-sensitized mice (section 4.3).

Overall, OP was shown to be hypoallergenic and weakly immunogenic *in vivo*, but to hold critical T cell epitopes that could help to prevent sensitization or restore tolerance to egg proteins. For its part, OA exhibited *in vivo* immunogenicity and primed mice for allergic reactions. However, mice administered OA+CT were not particularly prone to intestinal Th2 priming and subsequent adaptive responses as compared with those administered OP+CT. Therefore, in a subsequent step we explored the preventive potential of both hydrolysates.

4.4.1.2. The protective effect induced by the hydrolysate of OVA with pepsin was mediated by the expansion of T reg cells and the production of regulatory cytokines

Treatment with OVA, OA and OP for 3 weeks, before administration of EW+CT, prevented sensitization, as judged by significantly lower levels of serum EW-specific IgE, IgG2a and IgA

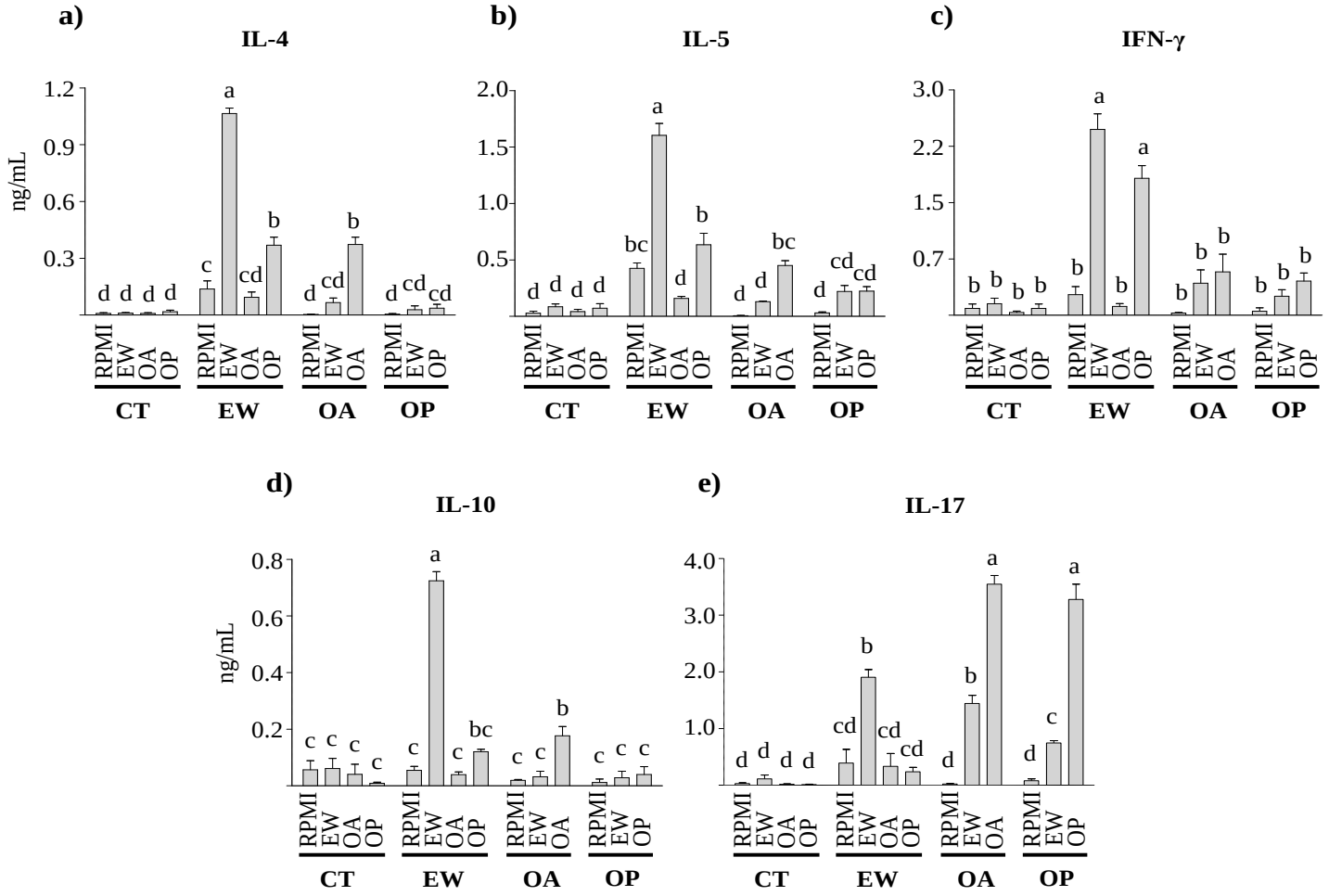

Figure 4.4.9. Effects of EW, OA and OP, on the secretion of IL-4 (a), IL-5 (b), IFN-γ (c) IL-10 (d) and IL-17 (e) by splenocytes from mice administered CT, or EW, OA and OP plus CT. RPMI culture medium is presented as negative control. Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

at the end of the experimental period, although only OP significantly decreased EW-specific IgG1 levels (Fig. 4.4.10). In addition, pre-treated mice did not experience anaphylaxis following oral challenge with EW, except for 2 out of 5 mice treated with OVA, which developed mild allergic signs. However, subsequent i.p. challenge led to significant clinical symptoms, temperature drops and release of mMCP-1 in mice previously treated with OVA and OA, while mice treated with OP were effectively protected against anaphylaxis (Fig. 4.4.11).

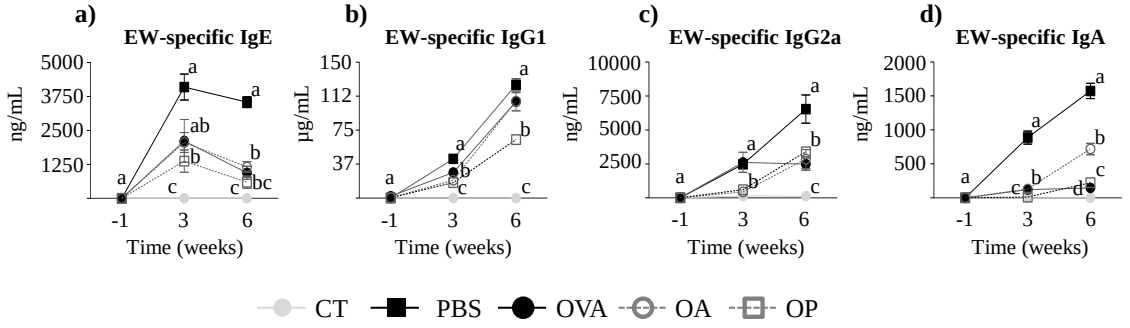

Figure 4.4.10. Preventive effect of intragastric administration of OVA, OA and OP on the antibody responses. Levels of EW-specific IgE (a), IgG1 (b), IgG2a (c) and IgA (d) were determined by ELISA in serum samples from CT (○), PBS (■), OVA (●), OA (○) and OP (□) groups collected at the end of the preventive treatment (week -1), during EW-sensitization period (week 3) and after challenge (week 6). Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$) between the groups for the same experimental point.

Pre-treatment of mice with OVA or OP before sensitization inhibited up-regulation of duodenal Th2-skewing cytokines following challenge with EW, except in the case of OA-treated mice, which showed expression levels of IL-33 similar to those seen in the untreated mice (Fig. 4.4.12). In general terms, prevention of sensitization and anaphylaxis by OVA and its hydrolysates was related to an increased expression of the regulatory cytokines IL-10 and TGF-β in the MLNs, as well as to upregulation of IL-17 (Fig. 4.4.13). This was accompanied by a decreased expression of GATA-3 in the PPs and MLNs of all the pre-treated animals (Fig. 4.4.14). In the groups pre-treated with OVA and OP, we also observed downregulation of T-bet in the duodenum and upregulation in the PPs. OP, which most effectively inhibited allergy development, distinctly upregulated IL-10 in PPs and TGF-β in duodenum and PPs, leading to a

significant increased expression of Foxp3 in intestinal lymphoid and non-lymphoid tissues and further increasing T-bet in the MLNs (Fig. 4.4.14).

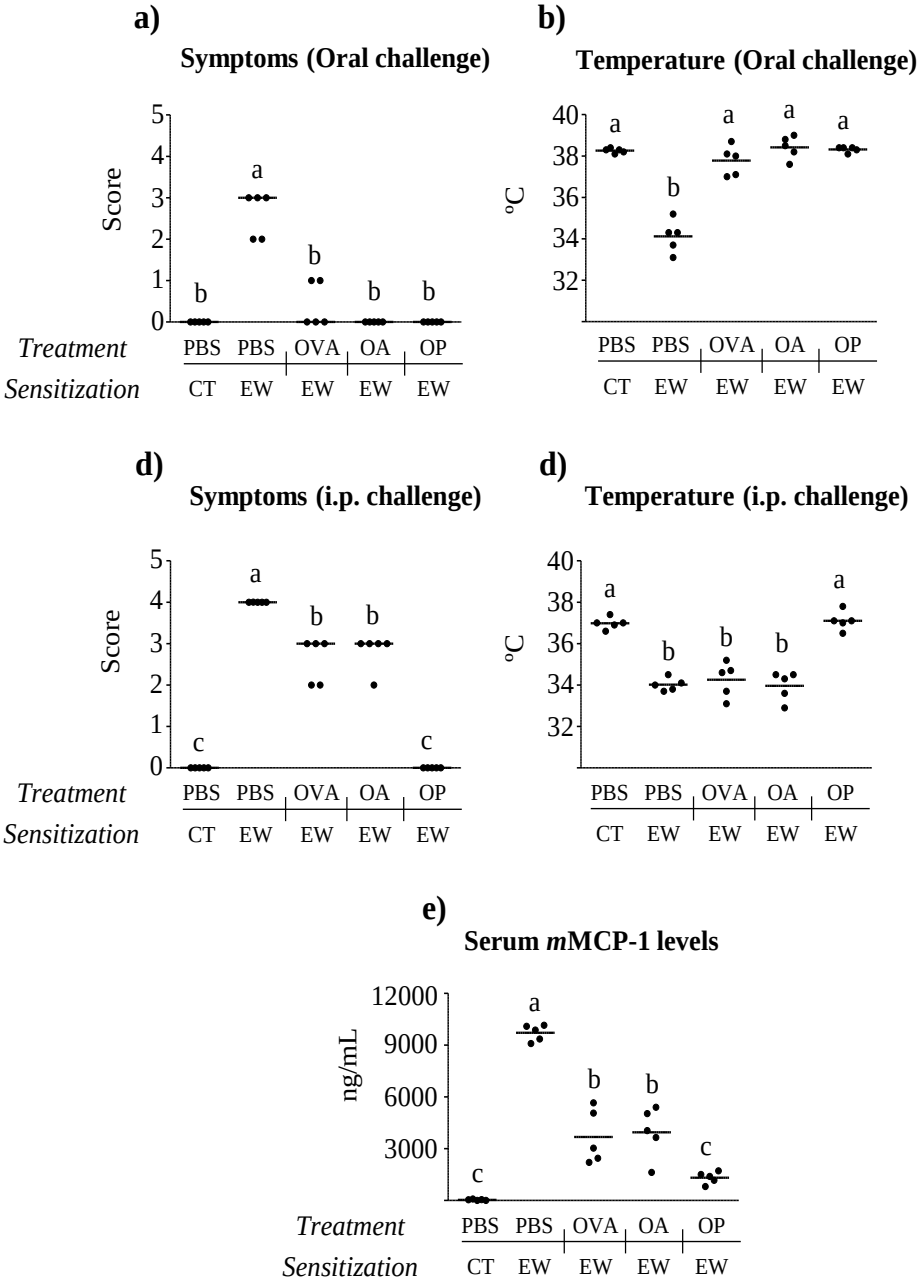

Figure 4.4.11. Effect of intragastric administration of OVA, OA and OP prior to EW sensitization on systemic anaphylactic responses after oral (a,b) and i.p. (c,d,e) challenge with EW. Anaphylactic score (a,c) and drop in body temperature (b,d) were determined 30 minutes after each challenge. Release of mMCP-1 (e) was quantified in serum samples collected after sacrifice (week 6). Data are represented individually for each mouse. Clinical scores are expressed as medians and, body temperature and levels of mMCP-1 expressed as means (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

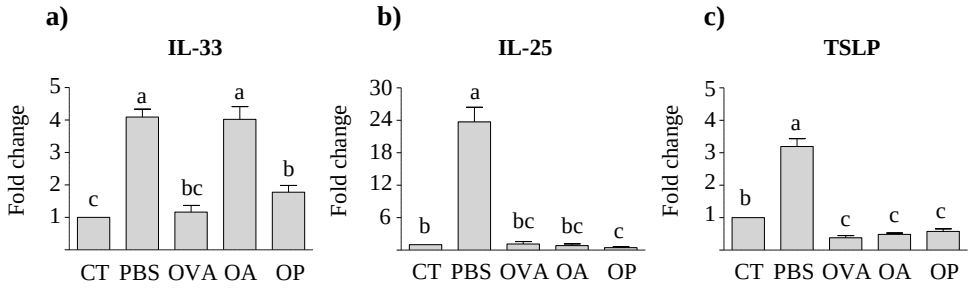

Figure 4.4.12. Modulation of intestinal expression, determined by RT-qPCR, of IL-33 (a), IL-25 (b) and TSLP (c) studied in duodenum samples from mice preventively treated with OVA, OA and OP. Results were normalized to β-actin and expressed relative to sham-sensitized group (identified as CT). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$).

All treatments reduced the percentage of activated splenic Th2 cells, although only OP concomitantly induced an increase in T reg cells (Fig. 4.4.15). Following incubation with the allergen, splenocytes from pre-treated mice, and particularly those from mice treated with OVA and OP, responded with significantly lower levels of IL-4, IL-5, IL-10 and IL-17 (Fig. 4.4.16). Furthermore, secretion of INF-γ was significantly decreased in the EW-stimulated spleen cell cultures from the OVA and OP pre-treated groups.

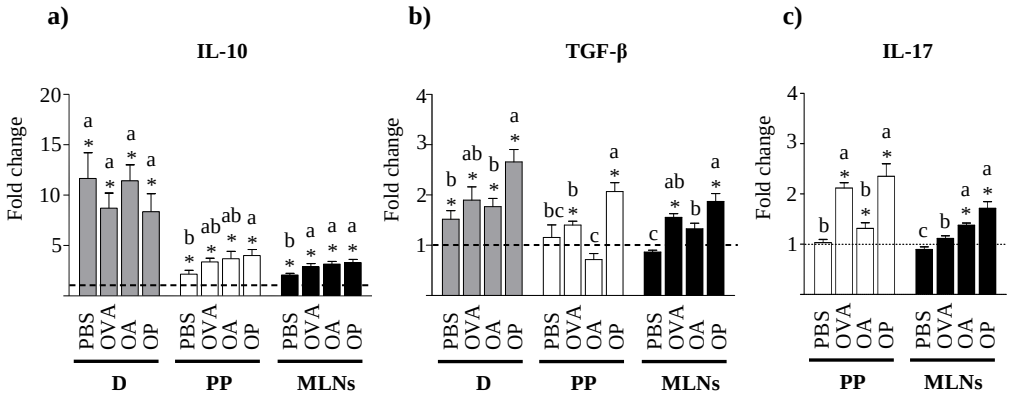

Figure 4.4.13. Modulation of relative gene expression, determined by RT-qPCR, of IL-10 (a), TGF-β (b) and IL-17 (c) studied in duodenum (D), PP and MLN samples from mice preventively treated with OVA, OA and OP. Results were normalized to β-actin and expressed relative to sham-sensitized group (relative expression of calibrator was 1 and it is represented as a discontinuous line in the figure). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$) within each organ and * indicates differences ($P < 0.05$) compared with the calibrator.

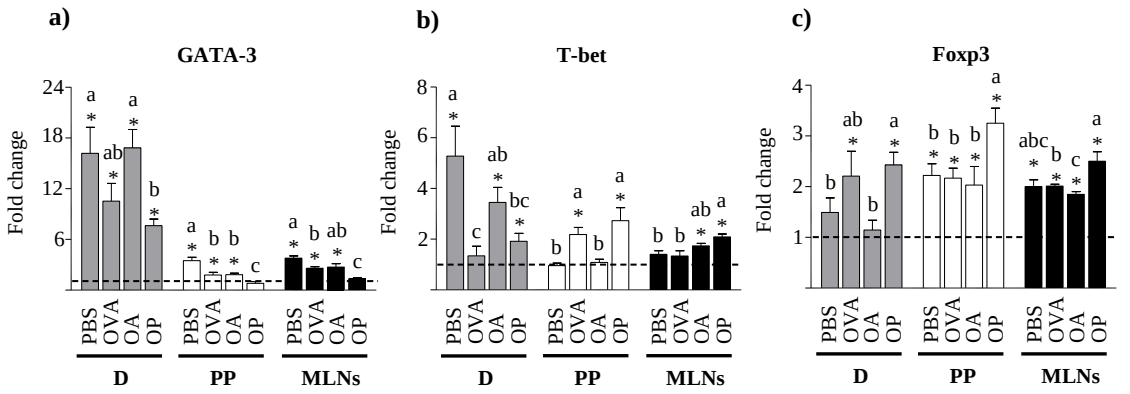

Figure 4.4.14. Modulation of relative gene expression, determined by RT-qPCR, of GATA-3 (a), T-bet (b) and Foxp3 (c) studied in duodenum (D), PP and MLN samples from mice preventively treated with OVA, OA and OP. Results were normalized to β-actin and expressed relative to sham-sensitized group (relative expression of calibrator was 1 and it is represented as a discontinuous line in the figure). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$) within each organ and * indicates differences ($P < 0.05$) compared with the calibrator.

These results indicate that pre-treatment with OP prevented the development of allergy to EW in mice through the enhancement of T reg cells, in contrast with OVA and OA, which did not avoid anaphylaxis following systemic challenge. Expression of the regulatory cytokines IL-10 and TGF-β, as well as IL-17, in intestinal tissues correlated with hindrance of sensitization and with clinical protection against anaphylaxis.

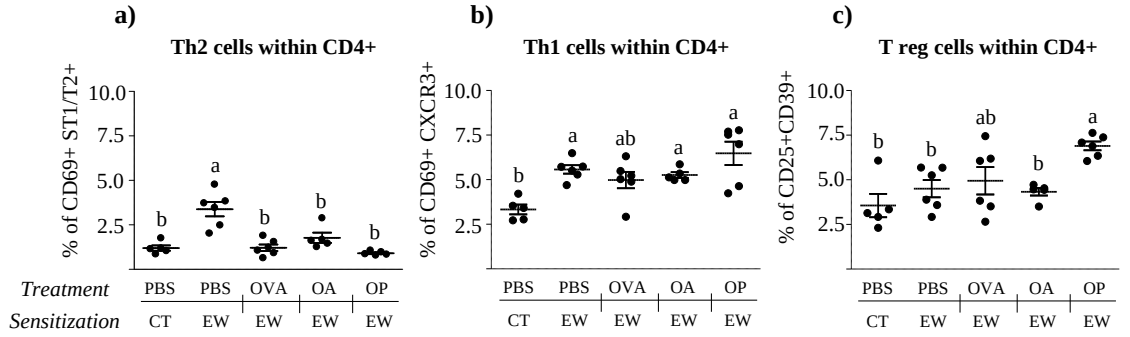

Figure 4.4.15. The percentages of activated Th2 (a), Th1 (b) and T reg (c) cells within the CD4+ population in spleens from mice preventively treated with OVA, OA and OP determined by flow cytometry. Individual values are represented for each mice and data expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

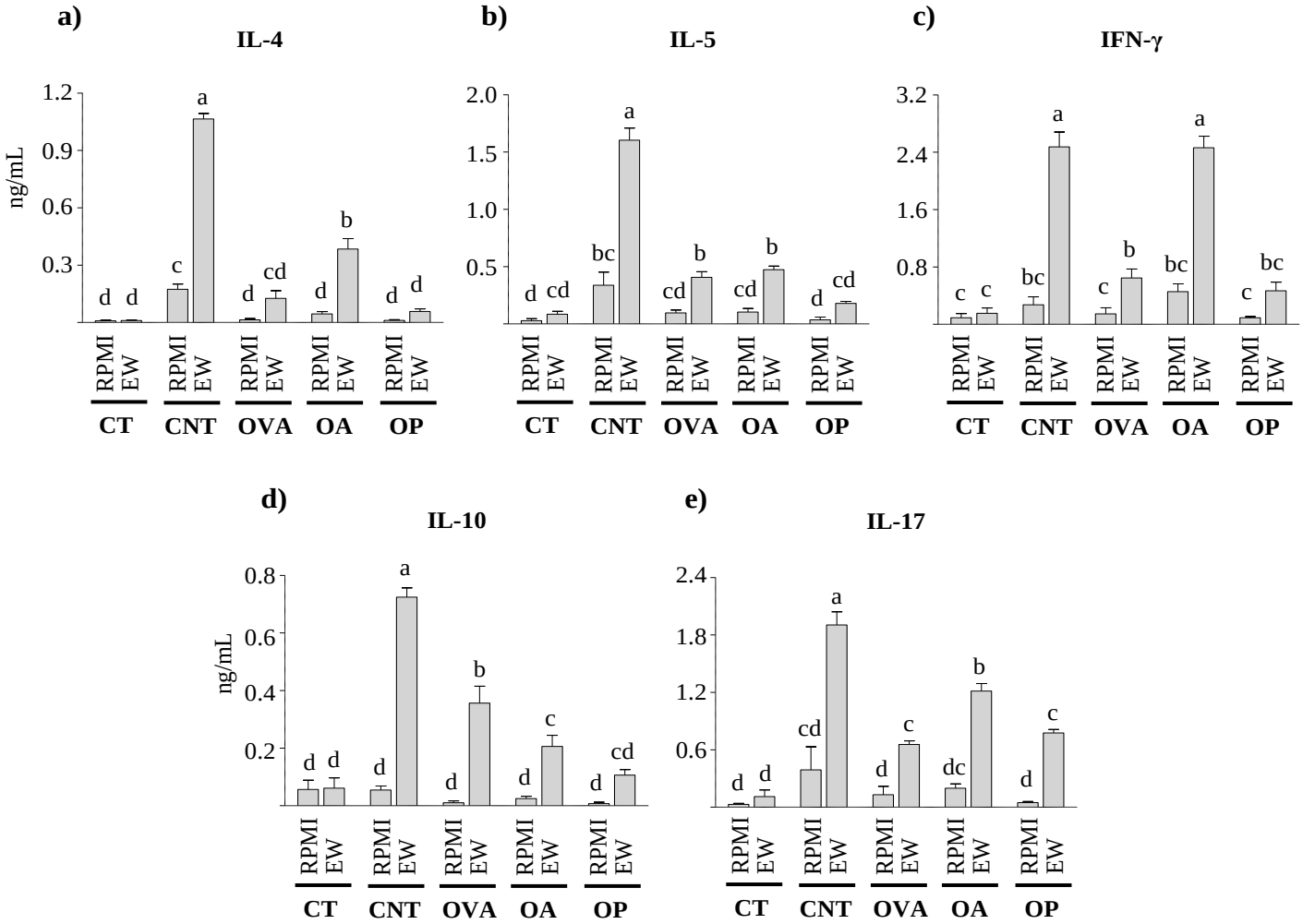

Figure 4.4.16. Effects EW on the secretion of IL-4 (a), IL-5 (b), IFN-γ (c) IL-10 (d) and IL-17 (e) by splenocytes from mice preventively treated with OVA, OA and OP. RPMI culture medium is presented as negative control. Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

4.4.1.3. The hydrolysate of OVA with pepsin offered a maintained therapeutic protection against allergy to EW more efficacious than intact OVA

In these experiments, once allergy to EW was stablished in mice by administration of EW plus CT, these were treated with OVA, OA and OP for 3 weeks and challenged with EW. Of note was the appearance of diarrhea, without other noticeable allergic signs, during the first week or OVA treatment (results not shown). Treatment with OP reduced significantly the levels of EW-specific IgE and IgG1, whereas neither OVA nor OA changed the serum concentration of these antibodies with respect to the untreated mice (Fig. 4.4.17a and b). In addition, none of the treatments altered the concentration of EW-specific IgG2a, while OVA, as well as OP, led to significantly increased levels of fecal IgA (Fig. 4.4.17c and d). Unlike treatment with OVA, which did not avoid anaphylaxis, the hydrolysates, OA and OP, abolished clinical signs and temperature changes upon oral challenge with EW (Fig. 4.4.18a and b). However, the subsequent i.p. challenge elicited allergy symptoms and temperature drops in OA treated mice and to a much lesser extent in OP treated mice. In agreement with the aforesaid, mMCP-1 levels were also the lowest in the OP treated animals, followed by those treated with OA (Fig. 4.4.18e).

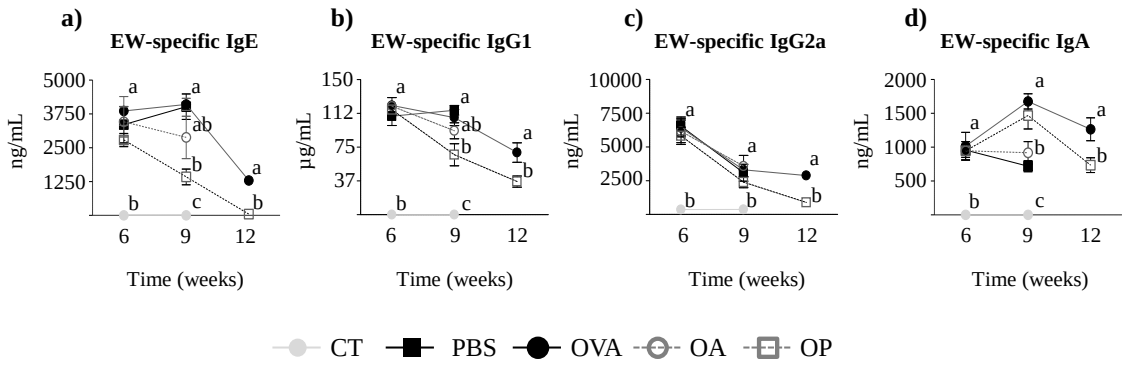

Figure 4.4.17. Therapeutic effect of intragastric administration of OVA, OA and OP on the antibody responses. Levels of EW-specific IgE (a), IgG1 (b), IgG2a (c) and IgA (d) were determined by ELISA in serum samples from CT (○), PBS (■), OVA (●), OA (○) and OP (□) groups collected at the end of the EW-sensitization period (week 6), three days after the end of the treatment (week 9) and 3 week off the therapy (week 12). Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$) between the groups for the same experimental point.

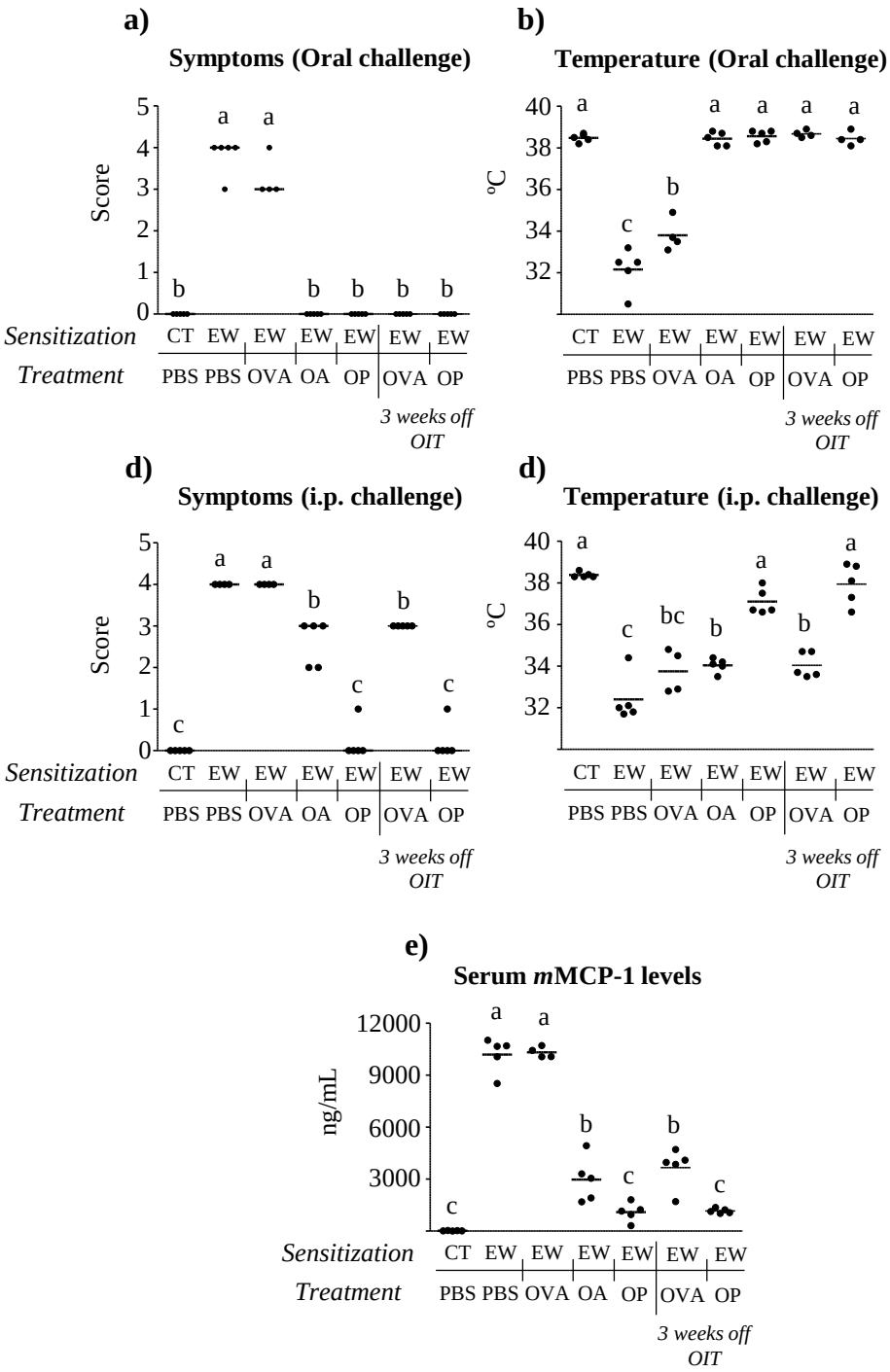

Figure 4.4.18. Therapeutic effect of intragastric administration of OVA, OA and OP to EW-sensitized mice on systemic anaphylactic responses after oral (a,b) and i.p. (c,d,e) challenge with EW. Anaphylactic score (a,c) and drop in body temperature (b,d) were determined 30 minutes after each challenge. Release of mMCP-1 (e) was quantified in serum samples collected after sacrifice (week 6). Data are represented individually for each mouse. Clinical scores are expressed as medians and, body temperature and levels of mMCP-1 expressed as means (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

Interestingly, reassessment of anaphylaxis 3 weeks after termination of the treatments with OVA and OP revealed similar responses in mice treated with OP, while those treated with OVA experienced no symptoms or temperature drop upon oral challenge and somehow milder changes (albeit not significantly different) upon subsequent i.p. challenge with EW (Fig. 4.4.18). These observations correlated with lower levels of IgE and IgG1, as compared with the point when both treatments were discontinued, and also lower concentrations of IgG2a and IgA in the case or OP (Fig. 4.4.17), showing sustained depletion of circulating and intestinal specific antibodies.

All the treatments reduced the expression of IL-33 and IL-25 in the duodenum induced by previous EW sensitization and challenge, to levels which, in the case of IL-25, were equivalent to those of sham-sensitized mice, while they did not change those of TSLP (Fig. 4.4.19). The therapeutic effect of OP could be traced to the increased expression of IL-10 and IL-17 in PPs and MLNs, and of TGF-β in all the intestinal tissues studied, observed in OP treated mice in comparison with OVA and OA treated mice (Fig. 4.4.20). In fact, treatment with OP distinctively increased the expression of Foxp3 in these intestinal tissues (Fig. 4.4.21). In particular, the least effective OVA treatment did not exert any effect on the expression of GATA-3 and T-bet in the MLNs, which might, at least partially, reflect the outcomes of challenge with EW in the animals treated with the intact protein; while in the animals treated with OP, as well as with OA, that did not experience symptoms after oral challenge, expression of GATA-3 and T-bet was decreased (Fig. 4.4.21).

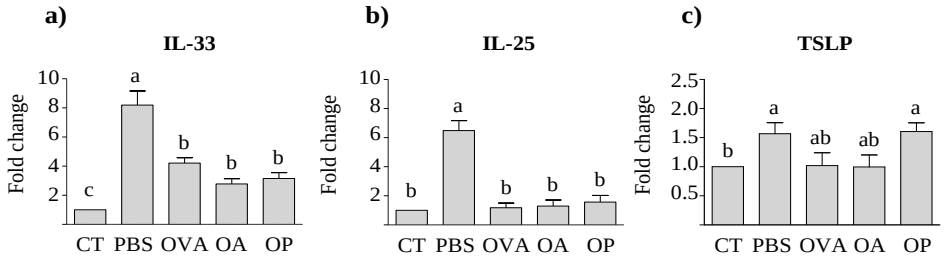

Figure 4.4.19. Modulation of intestinal expression, determined by RT-qPCR, of IL-33 (a), IL-25 (b) and TSLP (c) studied in duodenum samples from EW-sensitized mice treated with OVA, OA and OP. Results were normalized to β-actin and expressed relative to sham-sensitized group (identified as CT). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$).

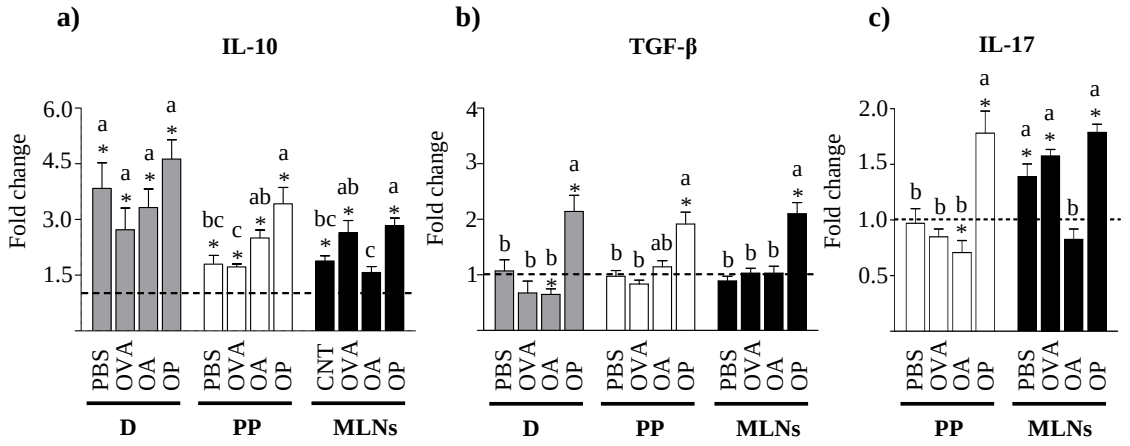

Figure 4.4.20. Modulation of relative gene expression, determined by RT-qPCR, of IL-10 (a), TGF-β (b) and IL-17 (c) studied in duodenum (D), PP and MLN samples from EW-sensitized mice treated with OVA, OA and OP. Results were normalized to β-actin and expressed relative to sham-sensitized group (relative expression of calibrator was 1 and it is represented as a discontinuous line in the figure). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$) within each organ and * indicates differences ($P < 0.05$) compared with the calibrator.

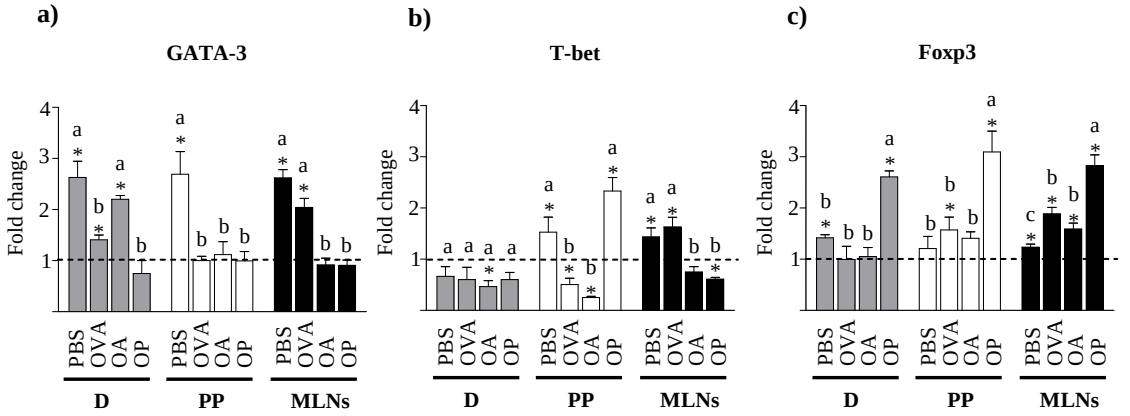

Figure 4.4.21. Modulation of relative gene expression, determined by RT-qPCR, of GATA-3 (a), T-bet (b) and Foxp3 (c) studied in duodenum (D), PP and MLN samples from EW-sensitized mice treated with OVA, OA and OP. Results were normalized to β-actin and expressed relative to sham-sensitized group (relative expression of calibrator was 1 and it is represented as a discontinuous line in the figure). Data are expressed as means ± SEM (n= 5). Different letters indicate statistically significant differences ($P < 0.05$) within each organ and * indicates differences ($P < 0.05$) compared with the calibrator.

Splenic activated Th2 cells were reduced in OVA and OP treated mice, whereas activated Th1 cells were unchanged (Figs. 4.4.22a and b). The percentage of T reg cells was specifically enhanced in the OP treated mice (Fig. 4.4.22c). Splenocytes from pre-treated mice, and particularly those from mice treated with OVA and OP, responded with a significantly

lower level of Th2 and Th1cytokines to EW stimulation. Noteworthy, EW-induced secretion of IL-10 and IL-17 from splenoctyes from OP treated mice was higher than that from the spleen cells from OVA and OA treated groups (Fig. 4.4.23). Hindrance of cytokine release from spleen cells upon EW stimulation was maintained in the OVA and OP groups 3 weeks after discontinuation of the respective treatments (Fig. 4.4.23).

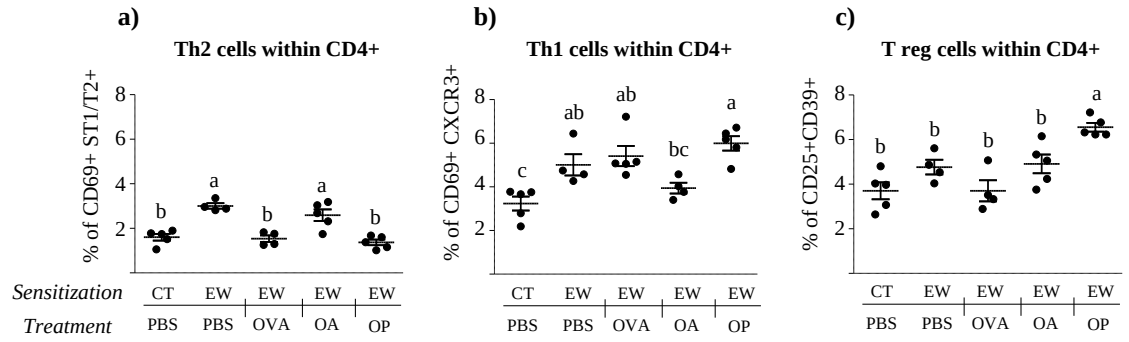

Figure 4.4.22. The percentages of activated Th2 (a), Th1 (b) and T reg (c) cells within the CD4+ population in spleens from EW-sensitized mice treated with OVA, OA and OP determined by flow cytometry. Individual values are represented for each mice and data expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

In general terms, OP provided effective immunotherapy with durable effects in EW allergic mice. Unlike OVA, OP decreased the levels of EW-specific IgE and IgG1 and hindered anaphylaxis upon oral and systemic changes. As it was the case of the preventive trials, intestinal T reg cells, IL-10, TGF-β, and IL-17 cells appeared as responsible for the curative effects observed.

4.4.2. Discussion

In this section, two hydrolysates of the egg allergen OVA, obtained by pepsin and alcalase treatment, were assayed for their ability to prevent allergy development and to achieve desensitization once allergy was stablished, using a mouse model of IgE-mediated EW hypersensitivity. Their suitability for prophylactic and therapeutic applications was compared with that of the intact allergen. The hydrolysates had to fulfil the requisite of lacking sensitizing and eliciting potential in EW-sensitized mice.

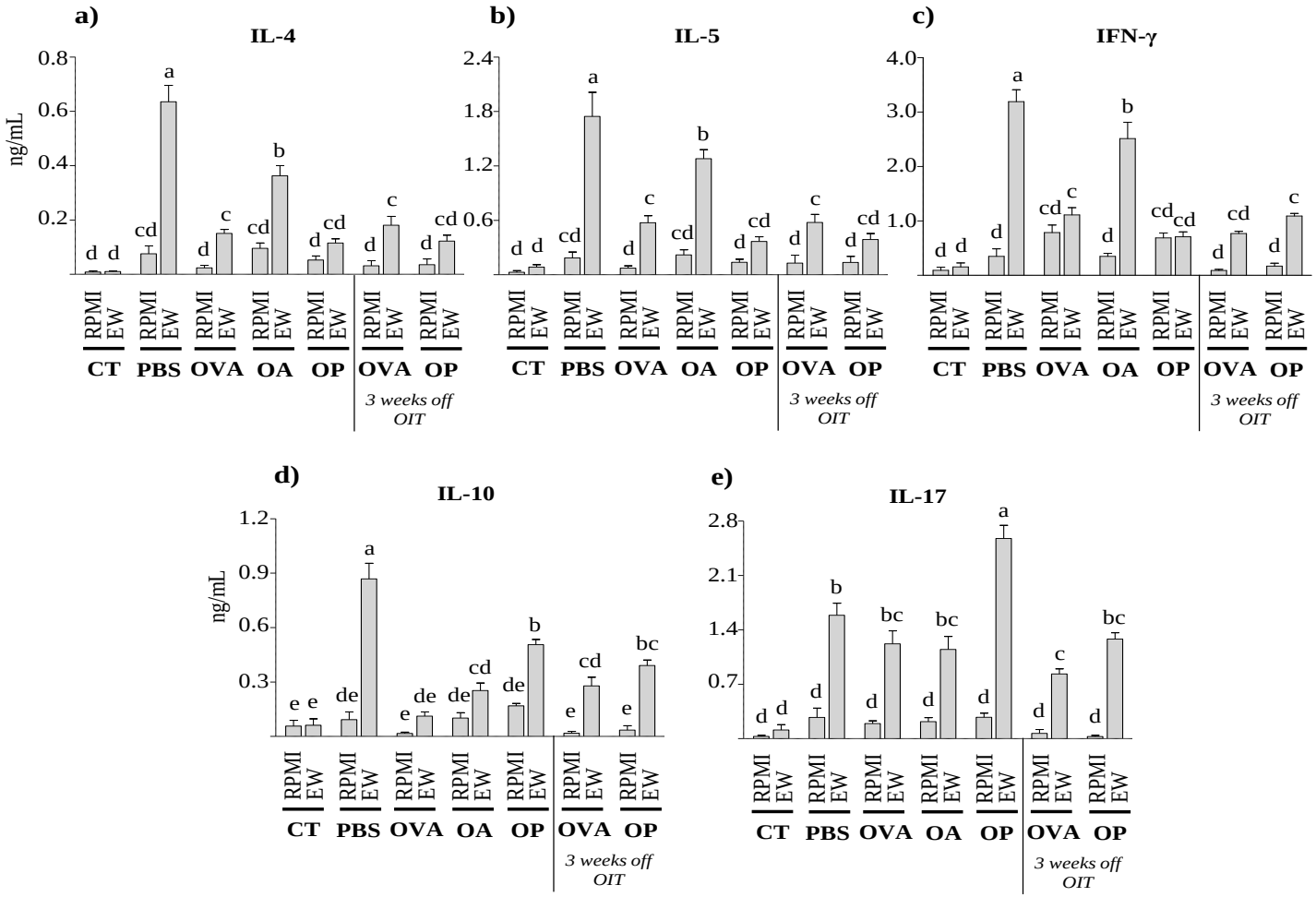

Figure 4.4.23. Effects of EW on the secretion of IL-4 (a), IL-5 (b), IFN-γ (c) IL-10 (d) and IL-17 (e) by splenocytes from EW-sensitized mice treated with OVA, OA and OP three hours and three weeks off OIT. RPMI culture medium is presented as negative control. Data are expressed as means ± SEM (n=5). Different letters indicate statistically significant differences ($P < 0.05$).

Unlike OP, OA induced antibody responses and allergic reactions in sensitized mice. These results were unexpected in view of the comparatively lower molecular mass of the peptides containing in OA in comparison with OP (with 85-97% versus 70% of the peptides lower than 1.5 kDa), the negligible binding of the former to IgE from allergic patients (sections 4.1 and 4.2) and the previous observation that none of the hydrolysates were able to trigger cutaneous allergic symptoms in PCA assays using serum of OVA+CT administered mice for passive sensitization (section 4.3). The assessment of the binding preferences of the antibodies raised in mice by OA+CT administration raised the question as to whether the enzyme preparation, and not just OVA-derived peptides, was the antigenic agent. Additionally, alcalase was also able to bind IgG1 antibodies raised in mice by EW-sensitization. In fact, it is documented that alcalase can induce specific antibodies, but also be recognized by antibodies arising from different environmental exposures (Sarlo et al., 2010). While it is likely that the OA components may have the ability to combine nonspecifically with the final products of immune responses, results from the preventive and therapeutic trials also suggest that they could stimulate IL-33 production at the intestinal mucosa and expansion of ILC2, as shown by the upregulated duodenal expression of GATA-3, that determines ILC2 differentiation, promoting Th2 responses (Noval Rivas et al., 2016). IL33 also activates mast cells, releasing mediators and playing a role in the progression of the allergic disease (Saluja et al., 2015). The observation that mice administered OA+CT experienced stronger anaphylactic reactions after oral challenge with OA as compared with the subsequent i.p. challenge could suggest that anaphylaxis is triggered in response to non-specifically driven signals arising from the intestinal epithelium. As a result, OA was not effective to induce oral tolerance to EW.

OP fulfilled the pre-requisite of lacking sensitizing capacity in order to be used to prevent allergy development. OP administered orally with CT was weakly immunogenic, because it did not induce specific antibodies, except for a negligible production of IgG2a, nor allergic sings upon oral or i.p. challenge. In addition, administration of OP for 3 weeks avoided subsequent sensitization to EW, as shown by inhibition of the generation of EW-specific IgE,

IgG1, IgG2a and IgA, allergic symptoms, body temperature changes and release of mast cell mediators. Partially hydrolyzed cow's milk proteins have been reported to deliver protective responses against milk allergy in mice (Fritsché et al., 1997; van Esch et al., 2011b). However, in certain cases, intact milk proteins were found to provide more effective protection against sensitization, linked to a more pronounced reduction in the levels of allergen specific IgE and/or IgG1 (Peng et al., 2004; Adel-Patient et al., 2011 and 2012; van Esch et al., 2011b). Nevertheless, in our study, unlike OP, pre-treatment with OVA did not hinder anaphylaxis following i.p. challenge with EW. In this respect, it should be noted that EW-specific serum IgE and IgG2a levels were similar in OP and OVA pre-treated mice, but EW-specific IgG1 was only significantly reduced in the former.

Anaphylaxis in the mouse has been described to occur through two independent pathways: the classical mechanism associated with human allergy, which involves the cross-linking of IgE bound to FcεRI on mast cells, and an alternative pathway that requires IgG antibodies, FcγRIII receptors and macrophages (Finkelman et al., 2016). Because much higher antibody and antigen concentrations are required to induce anaphylaxis by the alternative IgG-mediated pathway, parenteral administration of a relatively large quantity of antigen, as in our i.p. challenge tests, after CT-induced sensitization, largely drives IgG-mediated anaphylaxis that closely resembles that induced by IgE (Strait et al., 2006). Through PCA experiments with heated and unheated sera from EW-sensitized mice, we demonstrated that both specific IgE and IgG1 could participate in anaphylaxis in this model. Therefore, the allergic reactions observed after i.p. challenge in mice pre-treated with OVA are likely to be due to the lack of a significant lowering effect of the treatment on serum IgG1concentrations.

Our results relate the preventive effects of OVA and OP with a reduced expression in mouse intestinal tissues of the transcription factor GATA-3, that induces the differentiation of Th2 cells suppressing that of Th1 cells, in agreement with similar approaches using hydrolysates or peptides, which reported decreased numbers of activated Th2 cells in the MLNs following sensitization and challenge of mice (van Esch et al., 2011b). We also detected a

diminished proportion of activated Th2 cells in the spleen and decreased systemic Th2 responses upon stimulation of the splenocytes with the allergen used for sensitization, also in accordance with others (Adel-Patient et al., 2012; Hacini-Rachinel et al., 2014). Changes in the Th1 response were less consistent, with upregulated expression of T-bet in PPs and MLNs, but no change in the proportion of activated Th1 cells in the spleen and diminished allergen-stimulated production of IFN-γ. In parallel, with these immunologic changes, OP, unlike OVA, upregulated Foxp3, the specific marker of T reg cells that controls their differentiation and function, in the intestinal lymphoid tissues and increased the percentage of T reg cells in the spleen, in line with other studies that showed a key role of T reg cells induced at the MLN level in the prevention of sensitization and development of food allergy (Adel-Patient et al., 2011; van Esch et al., 2011b; Meulenbroek et al. 2013; Hacini-Rachinel et al., 2014).

OP was beforehand a good candidate to be used for allergen immunotherapy on the grounds of its lack of IgE reactivity (section 4.2), its reduced ability to trigger allergic symptoms in PCA assays and its potential to reduce splenic Th2 responses (release of IL-4 and IL-5) induced by egg allergens, but also to enhance Th1 responses (release of TNF-α and IFN-γ) (section 4.3). Assessment of the allergenicity of OP by challenge to mice sensitized to EW confirmed that it was hypoallergenic, even if there was a low degree of cross reactivity of IgG1 and IgG2a antibodies of EW-allergic mice to the hydrolysate, likely because oral administration of EW plus CT led to sensitization to *in vivo* pepsin digestion products of OVA.

As for its use as a therapeutic agent once EW allergy was established, OP decreased adverse reactions upon oral and subsequent i.p. challenges with the allergen. This correlated with a significant reduction in EW-specific IgE and IgG1 levels. However, treatment with the intact OVA for 3 weeks did not change the concentration of EW-specific IgE and IgG1, nor did it avoid allergic sings, temperature drops or release of mMCP-1 following challenge with EW. Successful desensitization with immunomodulatory hydrolysates or peptides is usually accompanied by a significant reduction in allergen-specific IgE levels (Yang et al., 2009b, 2010; Rupa and Mine, 2012; Wai et al., 2016). However, the role of other allergen-specific antibodies,

such as IgG and IgA is less clear. In view of the probable participation of IgG1 in anaphylaxis in our mouse model, decreased serum concentrations of this isotype are likely to account for clinical benefits. On the other hand, effective peptide-induced therapy has been attributed to increases in allergen-specific IgG2a or IgA levels, which would act as protective blocking antibodies in the circulation (Yang et al., 2010; Strait et al., 2011; Kulis et al., 2012; Rupa and Mine, 2012; Wai et al., 2016). In line with its weak immunogenicity, treatment with OP for 3 weeks did not increase the concentration of IgG2a although it enhanced fecal IgA. In fact, evidence for induction of blocking antibodies through peptide immunotherapy is scarce, because, unlike whole allergens, short peptides selected on the basis of low IgE-binding and anti-inflammatory properties hold a restricted potential to drive antibody production (Prickett et al., 2015; Valenta et al., 2016).

Whereas the role of blocking antibodies appears less important in peptide immunotherapy than in whole allergen immunotherapy; similarly to conventional immunotherapy, down-regulation of T cell proliferative and Th2 cytokine responses (whether IL-4, IL-5 or IL-13) to the allergen is usually reported (Yang et al., 2009b, 2010; Rupa and Mine, 2012; Kulis et al., 2012; Wai et al., 2016). However, different studies refer either an associated increase in Th1 cytokines (Yang et al., 2010; Rupa and Mine, 2012; Wai et al., 2016) or a concomitant Th1 decrease (Yang et al., 2009b). Treatment with OVA and OP decreased the expression of GATA-3 in the duodenum and PPs and the percentage of splenic activated Th2 cells, with no change in the percentage of activated Th1 cells. Furthermore, both treatments decreased the production of IL-4, IL-5, IFN-γ, IL10 and IL-17 upon splenocyte re-stimulation. OP additionally downregulated GATA-3 and T-bet in the MLN, thus showing that it more efficiently counterbalanced the immunostimulating effects of CT, which primes, not only Th2 (IL-4, IL-5 and IL-13), but also Th1 (IFN-γ) and Th17 (IL-17) responses in the MLNs and leads to mixed local and systemic cytokine responses (Blázquez and Berin, 2008). Upregulation of Foxp3 in intestinal tissues and of the percentage of T reg cells in the spleen of mice treated with OP points to a crucial role of these cells in the inhibition of Th1 and Th2 responses and

highlights induction of T reg cells as the main mechanism underlying the efficacy of tolerance induction by food peptides (Yang et al., 2009b and 2010; Wei et al., 2016).

While immunotherapy with peptides has demonstrated sustained tolerance with fewer adverse events than conventional allergen immunotherapy in clinical trials for cat, house dust mite and pollen allergy, it is in a much more exploratory stage in the field of food allergy, where it has almost exclusively been assayed *in vivo* in murine models (Berin, 2014). Moreover, almost no studies compared the therapeutic efficacy of food peptides or hydrolysates with that of intact allergens or re-assessed protection from anaphylaxis after discontinuation of the treatment.

According to Kulis et al. (2012), immunotherapy with pepsin-hydrolyzed cashew, which maintains the immunogenicity of the parent protein and its sensitizing potential but lacks eliciting potential, works through the same mechanism as immunotherapy with the intact allergen by virtue of the strong Th2-stimulating properties of the hydrolyzate. However, in our study, the immunomodulating peptides contained in OP, which were selected on the basis of their Th1 stimulating properties and absence of Th2 inducing capacity (section 4.2 and 4.3) were more effective that OVA in the induction of tolerance. In addition, repeated OVA gavage during immunotherapy treatment, unlike OP gavage, would have worked as a low-level repeated intragastric challenge, releasing epithelium-derived cytokines, such as IL-33 and IL-25. Furthermore, although no anaphylactic responses were observed, diarrhea during the first week of treatment with OVA was a symptomatic feature of IgE-mediated reactions. Stimulation of mouse mast cells by IgE cross-linking releases Th2-driving cytokines, such as IL-25, and enhances IL-4, acting as an amplifier of Th2 responses (Ikeda et al., 2003; Burton et al., 2014). Thus, repeated intragastric challenge induces intestinal IL-33, IL-25 and TSLP signaling and enhances Th2 cells, promoting the expansion of mucosal ILC2 and the collaboration between these innate cells and allergen-specific Th2 cells, ultimately acting, through IL-4, as a Foxp3 inhibitor in T cell differentiation (Lee et al., 2016; Noval Rivas et al., 2016). Therefore, the lack

of IgE-binding and Th2-priming capacity of OP not only increases safety, but also efficacy of immunotherapy.

Nevertheless, both OVA and OP treatments, offered long term desensitization. In fact, protection generated by OP was sustained 3 weeks after discontinuation of the treatment and that induced by OVA even increased after that period. This observation differs from the findings of Leonard et al. (2012) who reported that EW, administered as immunotherapy to OVA sensitized mice induced clinical, protection through desensitization but not immune tolerance. The immunological mechanism that appears to provide a long lasting beneficial effect of OVA an OP is related to the induction of T reg cells. Furthermore, it is noteworthy that peptides resulting from OVA fragmentation offered protection against challenge with whole EW. In this respect, it is also hypothesized that the most effective way to provide tolerance towards multiple epitopes within an allergenic protein, or even towards other allergens normally co-exposed with it, is through the induction of a regulatory function in the T cells primed by the tolerogenic substance (Mackenzie et al., 2012).

The superior potential of OP versus OVA for allergy prevention and therapy linked to T reg cell development parallels a more efficient upregulation of the cytokines TGF-β, IL-10 and IL-17 in the intestinal tissues. Different cells, including DCs and IECs, express TGF-β and IL-10 that facilitate the generation of T reg cells. In turn, T reg cells themselves produce TGF-β and IL-10 as part of their suppressive role on the conversion of naïve T cells into allergen-specific Th2 cells and on the production of IL-4 (Palomares et al., 2014; Noval Rivas and Chatila, 2016) that, in mice, induces switching to IgE and IgG1 (Mestas and Hughes, 2004). TGF-β is a very important immunosuppressive cytokine through its critical function as a direct antagonist of Th1 development, affecting IFN-γ and T-bet, as well as of Th2 differentiation, but also as an inducer of T reg cells. However, an inflammatory stimulus, such as IL-6, suppresses the generation of TGF-β-induced T reg cells, allowing the diversion to IL-17 producing T cells, a process that, in the context of allergic diseases, can redirect a regulatory response towards a pro-inflammatory pathogenic phenotype (Bettelli et al., 2006; Veldhoen et al., 2006; Massoud et

al., 2016). Thus, T reg and Th17 cells arise in the periphery through a common intermediate that simultaneously upregulates both Foxp3 and RORγt, the Th17 master transcription factor, which in the mice represents a stable, suppressor effector T reg lineage, and competition of T reg with Th17 for their reciprocal development from this common precursor controls T reg or Th17 development (Lochner et al., 2015). Furthermore, it is now known that the microbiota induces T reg cells bearing RORγt that differentiate along a pathway that also leads to Th17 cells, and that both, RORγt+ T reg and Th17 cells, contribute to the control of exacerbated Th2 responses (Ohnmacht et al., 2015; Chinthrajah et al., 2016). In fact, T reg cells not only suppress the function of Th17 cells, but also support their development, since low levels of IL-17 in the intestine are beneficial and protect from inflammation (Lochner et al., 2015). Therefore, the concomitant upregulation of TGF-β, IL-10 and IL-17 observed in the intestinal tissues of mice, either preventive or therapeutically treated with OP, could work together in the inhibition of Th2 and inflammatory responses contributing to a balanced immune response.

In conclusion, OP was hypoallergenic and weakly immunogenic and offered preventive and therapeutic protection against allergy to EW through the induction of T reg cells and the upregulation of TGF-β, IL-10 and IL-17 in intestinal tissues. This restrained the differentiation of Th2 cells and led to low Th2 and Th1 systemic responses following allergen stimulation. OP was more efficacious than intact OVA in desensitizing mice by virtue of its lower allergenicity and its immunomodulating capacity. In general terms, our screening strategy proved useful to select the critical hydrolysate that combined desired tolerance with the absence of undesired effects of IgE-crosslinking and inflammatory cell activation, although *in vivo* testing of OA revealed immunogenic and antigenic capacities not previously detected by *in vitro* methods. This study provides evidence for the essential role of T reg in the prevention of allergy development and in the promotion of long term desensitization to allergens as well as of linked-epitope suppression.

5. CONCLUSIONS / CONCLUSIONES

Based on the results obtained in this thesis, the following conclusions can be drawn:

First: The hydrolysates of egg white proteins produced with alcalase, and that of ovalbumin with pepsin, are hypoallergenic revealing a low IgE-binding capacity demonstrated *in vitro* and *in vivo.*

Second: The enzymatic treatment of egg white proteins with alcalase, together with the ovalbumin hydrolysed with pepsin, contain modulating peptides able to decrease the release of Th2-biased cytokines and inhibit inflammatory events in murine cells unspecifically stimulated with T and B cells mitogens.

Third: The hydrolysates prepared with alcalase, and that of ovalbumin with pepsin, help to re-establish the Th1/Th2 balance in a model of Th2-skewed peripheral blood mononuclear cells. In addition, these hydrolysates also inhibit the release of pro-inflammatory mediators and reduce oxidative stress in peripheral blood leukocytes treated with inflammatory stimuli.

Fourth: The hydrolysate of ovalbumin with pepsin shows immunomodulatoting effects on splenocytes from BALB/c mice sensitized to individual egg proteins, or their mixture in different proportion. This hydrolysate stimulates slight immune responses and down-regulates Th2 cytokine profile induced by intact egg allergens *ex vivo*, contributing to increase the secretion of Th1-biased cytokines.

Fifth: Ovalbumin hydrolysed with alcalase induces low Th2 responses in spleen cells from mice sensitized to egg white proteins in different proportions. However, this hydrolysate reveals immunogenic properties when it is administered together with cholera toxin to BALB/c mice and an antigenic ability offered to a murine model of egg allergy.

Sixth: The hydrolysate of ovalbumin with pepsin is weakly immunogenic and offers preventive and therapeutic protection against allergy to egg white through the induction of regulatory T cells and the upregulation of TGF-β, IL-10 and IL-17 in intestinal tissues.

Seventh: The therapeutic administration of ovalbumin hydrolysed with pepsin is more efficacious in egg white-sensitized mice than the intact protein it derives from, by virtue of its lower allergenicity and its immunomodulating capacity.

Finally, in the light of the results achieved, the screening strategy followed in this work proved useful to select the ovalbumin enzymatically treated with pepsin as a critical hydrolysate that combines tolerance induction with the absence of undesired effects of IgE-crosslinking and inflammatory cell activation. Together, this doctoral thesis stands the hydrolysate of ovalbumin with pepsin as a simple to obtain, cheap and safe candidate to be used in the prevention and treatment of egg allergy.

A partir de los resultados obtenidos en esta tesis, se pueden extraer las siguientes conclusiones:

Primera: Los hidrolizados de clara de huevo producidos con alcalasa, y el de ovalbúmina con pepsin, son hipoalergénicos presentando una baja capacidad de unión a IgE que se demostró tanto *in vitro* como *in vivo.*

Segunda: El tratamiento de las proteínas de la clara de huevo con alcalasa, junto con la ovalbúmina hidrolizada con pepsin, contienen péptidos modulantes capaces de disminuir la liberación de citoquinas del tipo Th2 y de inhibir eventos inflamatorios en células de ratón inespecíficamente estimuladas con mitógenos de células T y B.

Tercera: Los hidrolizados preparados con alcalasa, y el de ovalbúmina con pepsina, ayudan a restablecer el balance Th1/Th2 en un modelo de células mononucleares humanas de sangre periférica polarizadas hacia una respuesta Th2. Además, estos hidrolizados también inhiben la liberación de mediadores pro-inflamatorios en leucocitos humanos de sangre periférica derivados hacia una respuesta del tipo Th1.

Cuarta: La ovalbúmina hidrolizada con pepsina muestra efectos inmunomodulantes en esplenocitos de ratones BALB/c sensibilizados a las proteínas del huevo, individualmente o en su mezcla a diferentes proporciones. Este hidrolizado produce una débil estimulación de la respuesta inmune a la vez que es capaz de disminuir el perfil de citoquinas Th2 inducido por el propio alérgeno *ex vivo* mediante el aumento de la secreción de citoquinas del tipo Th1.

Quinta: El hidrolizado de ovalbúmina con alcalasa induce una escasa respuesta Th2 en células de bazo procedentes de ratones sensibilizados a las proteínas de la clara de huevo, individualmente o en diferentes proporciones. Sin embargo, este hidrolizado posee propiedades inmungénicas cuando es administrado junto con toxina de cólera a ratones BALB/c y muestra un marcado carácter antigénico cuando es suministrado a un modelo múrido de alergia al huevo.

Sexta: La ovalbúmina hidrolizada con pepsina es escasamente inmunogénica y confiere protección, tanto preventiva como terapéutica, frente a la alergia al huevo a través de la inducción de células T reguladoras y la producción de TGF-β, IL-10 e IL-17 a nivel intestinal.

Séptima: La administración terapéutica del hidrolizado de ovalbúmina con pepsina es más eficaz que el uso de la proteína intacta de la que proviene debido a su menor alergenicidad y a su capacidad inmunomodulante.

Finalmente, a la luz de estos resultados, se puede establecer que la estrategia de evaluación seguida en este trabajo fue útil para la selección de la ovalbúmina hidrolizada con pepsina como una preparación que combina la capacidad de inducir tolerancia con la ausencia de efectos indeseables derivados del entrecruzamiento de las moléculas de IgE o de la activación inflamatoria a nivel celular. En su conjunto, esta tesis doctoral revela al hidrolizado de ovalbúmina con pepsina como un posible candidato sencillo de obtener, barato y seguro para ser utilizado en la prevención y el tratamiento de la alergia al huevo.

6. REFERENCES

Alberse, R.C., and Crameri, R. (2011). Ig-E binding epitopes: a reappraisal. *Allergy*. **66**:1261-1274.

Adel-Patient, K., Wavrin, S., Bernard, H., Meziti, N., Ah-Leung, S., and Wal, J.M. (2011). Oral tolerance and Treg cells are induced in BALB/c mice after gavage with bovine β-lactoglobulin. *Allergy*. **66**: 1312-1321.

Adel-Patient, K., Nutten, S., Bernard, H., Fritsché, R., Ah-Leung, S., Meziti, N., Prioult, G., Mercenier, A., and Wal, J.M. (2012). Immunomodulatory potential of partially hydrolyzed β-lactoglobulin and large synthetic peptides. *J. Agric. Food Chem.* **60**: 10858-10866.

Ahn, C.B., Cho, Y.S., and Je, J.Y. (2015). Purification and anti-inflammatory action of tripeptide from salmon pectoral fin byproduct protein hydrolysate. *Food Chem.* **168**: 151-156.

Akdis, M., and Akdis, C.A. (2014). Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J. Allergy Clin. Immunol*. **133**: 621-631.

Albrecht, M., Kühne, Y., Ballmer-Weber, B.K., Becker, W.M., Holzhauser, T., Lauer, I., Reuter, A., Randow, S., Falk, S., Wangorsch, A., Lidholm, J., Reese, G., and Vieths, S. (2009). Relevance of IgE binding to short peptides for the allergenic activity of food allergens. *J. Allergy Clin. Immunol*. **124**: 328-336.

Almqvist, N., Lönnqvist, A., Hultkrantz, S., Rask, C., and Telemo, E. (2008). Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma. *Immunology*. **125**: 21–27.

Archila, L.D., Jeong, D., Pascal, M., Bartra, J., Juan, M., Robinson, D., Farrington, M.L., and Kwok, W.W. (2015). Jug r 2-reactive CD4(+) T cells have a dominant immune role in walnut allergy. *J. Allergy Clin. Immunol*. **136**: 983-992.

Bamdad, F., Ahmed, S., and Chen, L. (2015). Specifically designed peptide structures effectively suppressed oxidative reactions in chemical and cellular systems. *J. Funct. Foods*. **18**: 35-46.

Baravalle, G., Greer, A.M., LaFlam, T.N., and Shin, J.S. (2014). Antigen-conjugated human IgE induces antigen-specific T cell tolerance in a humanized mouse model. *J. Immunol*. **192**: 3280-3288.

Barnes, P.J. (2011). Pathophysiology of allergic inflammation. *Immunol. Rev*. **242**: 31-50.

Benedé, S., López-Fandiño, R., Reche, M., Molina, E., and López-Expósito, I. (2013). Influence of the carbohydrate moieties on the immunoreactivity and digestibility of the egg allergen ovomucoid. *PLoS One*. **8**: e80810.

Benedé, S., López-Expósito, I., López-Fandiño, R., and Molina, E. (2014). Identification of IgE-binding peptides in hen egg ovalbumin digested in vitro with human and simulated gastroduodenal fluids. *J. Agric. Food Chem.* **62**: 152-158.

Benedé, S., López-Expósito, I., Molina, E., and López-Fandiño, R. (2015). Egg proteins as allergens and the effects of the food matrix and processing. *Food Funct.* **6**: 694-713.

Becker, K., Schroecksnadel, S., Gostner, J., Zaknun, C., Schennach, H., Uberall, F., and Fuchs, D. (2014). Comparison of in vitro tests for antioxidant and immunomodulatory capacities of compounds, *Phytomedicine*. **21**: 164-171.

Berin, M.C. (2014). Future therapies for IgE-mediated food allergy. *Curr. Pediatr. Rep*. **2**: 119-126.

Berin, M.C. (2015). Pathogenesis of IgE-mediated food allergy. *Clin. Exp. Allergy*. **45**: 1150-1161.

Berin, M.C., and Sampson, H.A. (2013). Mucosal immunology of food allergy. *Curr. Biol*. **23:** 389-400.

Berin, M.C., and Shreffler, W.G. (2008). Th2 adjuvants: Implications for food allergy. *J. Allergy Clin. Immunol*. **121**: 1311-1320.

Bernasconi, E., Fritsché, R., and Corthésy, B. (2006). Specific effects of denaturation, hydrolysis and exposure to Lactococcus lactis on bovine beta-lactoglobulin transepithelial transport, antigenicity and allergenicity. *Clin. Exp. Allergy*. **36**: 803-814.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. **441**: 235-238.

Bischoff, S.C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.D., Serino, M., Tilg, H., Watson, A., and Wells, J.M. (2014). Intestinal permeability - a new target for disease prevention and therapy. *BMC Gastroenterol*. **14**: 189.

Biziulevicius, G.A., Kislukhina, O.V., Kazlauskaite, J., and Zukaite, V. (2006). Food-protein enzymatic hydrolysates possess both antimicrobial and immunostimulatory activities: a "cause and effect" theory of bifunctionality. *FEMS Immunol. Med. Microbiol*. **46**: 131-138.

Blázquez, A.B., and Berin, M.C. (2008). Gastrointestinal dendritic cells promote Th2 skewing via OX40L. *J. Immunol*. **180**: 4441-50.

Bøgh, K.L., Kroghsbo, S., Dahl, L., Rigby, N.M., Barkholt, V., Mills, E.N., and Madsen, C.B. (2009). Digested Ara h 1 has sensitizing capacity in Brown Norway rats. *Clin. Exp. Allergy*. **39**:1611-1621.

Bøgh, K.L., Barkholt, V., Rigby, N.M., Mills, E.N., and Madsen, C.B. (2012). Digested Ara h 1 loses sensitizing capacity when separated into fractions. *J. Agric. Food Chem*. **60**: 2934-2942.

Bøgh, K.L., Barkholt, V., and Madsen, C.B. (2013). The sensitizing capacity of intact β-lactoglobulin is reduced by co-administration with digested β-Lactoglobulin. *Int. Arch. Allergy Immunol.* **161**: 21-36.

Bøgh, K.L., Barkholt, V., and Madsen, C.B. (2015). Characterization of the Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates-A Study in Brown Norway Rats. *Scand. J. Immunol*. **81**: 274-283.

Bohle, B., Radakovics, A., Jahn-Schmid, B., Hoffmann-Sommergruber, K., Fischer, G.F., and Ebner, C. (2003). Bet v 1, the major birch pollen allergen, initiates sensitization to Api g 1, the major allergen in celery: evidence at the T cell level. *Eur. J. Immunol*. **33**: 3303-3310.

Bohle, B., Radakovics, A., Luttkopf, D., Jahn-Schmid, B., Vieths, S., and Ebner, C. (2005). Characterization of the T cell response to the major hazelnut allergen, Cor a 1.04: evidence for a relevant T cell epitope not cross-reactive with homologous pollen allergens. *Clin. Exp. Allergy*. **35**: 1392-1399.

Brix, S., Bovetto, L., Fritsché, R., Barkholt, V., and Frøkiaer, H. (2003). Immunostimulatory potential of β-lactoglobulin preparations: Effects caused by endotoxin contamination. *J. Allergy Clin. Immunol.* **112**: 1216-1222.

Burks, A.W., Laubach, S., and Jones, S.M. (2008). Oral tolerance, food allergy, and immunotherapy: implications for future treatment. *J Allergy Clin Immunol*. **121**: 1344-50.

Burton, O.T., Noval Rivas, M., Zhou, J.S., Logsdon, S.L., Darling, A.R., Koleoglou, K.J., Roers, A., Houshyar, H., Crackower, M.A., Chatila, T.A., and Oettgen, H.C. (2014). Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. *Immunity*. **41**: 141-151.

Capozzi, A., Vincentini, O., Gizzi, P., Porzia, A., Longo, A., Felli, C., Mattei, V., Mainiero, F., Silano, M., Sorice, M., and Misasi, R. (2013). Modulatory Effect of Gliadin Peptide 10-mer on Epithelial Intestinal CACO-2 Cell Inflammatory Response. *PLoS ONE.* **8**: e66561.

Cardoso, C. R., Provinciatto, P. R., Godoi, D. F., Ferreira, B. R., Teixeira, G., Rossi, M. A., Cunha, F. Q., and Silva, J. S. (2009). IL-4 regulates susceptibility to intestinal inflammation in murine food allergy. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **296**: G593-600.

Casale, T.B., and Stokes, J.R. Future forms of immunotherapy. *J. Allergy Clin. Immunol.* **127**: 8-15.

Chalamaiah, M., Hemalatha, R., Jyothirmayi, T., Diwan, P.V., Kumar, P.U., Nimgulkar, C., and Kumar, B.D. (2014). Immunomodulatory effects of protein hydrolysates from rohu (Labeo rohita) egg (roe) in BALB/c mice. *Food Res. Int.* **62**: 1054-1061.

Chalamaiah, M., Hemalatha, R., Jyothirmayi, T., Diwan, P.V., Bhaskarachary, K., Vajreswari, A., and Kumar, B,D. (2015). Chemical composition and immunomodulatory effects of enzymatic protein hydrolysates from common carp (Cyprinus carpio) egg. *Nutrition.* **31**: 388-398.

Cheng, M.L., Wang, H.C., Hsu, K.C., and Hwang, J.S. Anti-inflammatory peptides from enzymatic hydrolysates of tuna cooking juice. *Food Agric. Immunol*. **26**: 770-781.

Chicón, R., Belloque, J., Recio, I., and López-Fandiño, R. (2006). Influence of high hydrostatic pressure on the proteolysis of beta-lactoglobulin A by trypsin. *J. Dairy Res.* **73**: 121-8.

Chinthrajah, R.S., Hernandez, J.D., Boyd, S.D., Galli, S.J., and Nadeau, K.C. (2016). Molecular and cellular mechanisms of food allergy and food tolerance. *J. Allergy Clin. Immunol.* **137**: 984-97.

Chu, D.K., Llop-Guevara, A., Walker, T.D., Flader, K., Goncharova, S., Boudreau, J.E., Moore, C.L., Seunghyun, In T., Waserman, S., Coyle, A.J., Kolbeck, R., Humbles, A.A., and Jordana, M. (2013). IL-33, but not thymic stromal lymphopoietin or IL-25, is central to mite and peanut allergic sensitization. *J. Allergy Clin. Immunol*. **131**: 187-200.

Cian, R.E., López-Posadas, R., Drago, S.R., Sánchez de Medina, F., and Martínez-Augustin, O. (2012). A Porphyra columbina hydrolysate upregulates IL-10 production in rat macrophages and lymphocytes through an NF-κB, and p38 and JNK dependent mechanism. *Food Chem*. **134**: 1982-1990.

Claustre, J., Toumi, F., Trompette, A., Jourdan, G., Guignard, H., Chayvialle, J.A., and Plaisancié, P. (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. *Am. J. Physiol. Gastrointest. Liver Physiol*. **283**: G521-528.

Commission Directive 96/4/EC of 16th February 1996 amending Directive 91/321/EEC on infant formulae and follow-on formulae. *Official Journal of the European Communities.* No L **49**: 12-16.

Coombes, J.L., Siddiqui, K.R., Arancibia-Cárcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J. Exp. Med.* **204**: 1757-1764.

Cosentino, M., Bombelli, R., Carcano, E., Luini, A., Marino, F., Crema, F., Dajas, F., and Lecchini, S. (2008). Immunomodulatory properties of Achyrocline satureioides (Lam.) D.C. infusion: a study on human leukocytes. *J. Ethnopharmacol.* **116**: 501-507.

Daddaoua, A., Puerta, V., Zarzuelo, A., Suárez, M.D., Sánchez de Medina, F., and Martínez-Augustin, O. (2005). Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *J. Nutr*. **135**: 1164-1170.

Dávalos, A., Miguel, M., Bartolomé, B., and López-Fandiño, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *J. Food Prot.* **67**: 1939-1944.

de Mejía, E.G., and Dia, V.P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-kappaB pathway in the macrophage. *Peptides*. **30**: 2388-2398.

DeLong, J.H., Simpson, K.H., Wambre, E., James, E.A., Robinson, D., and Kwok, W.W. (2011). Ara h 1-reactive T cells in individuals with peanut allergy. *J. Allergy Clin. Immunol.* **127**: 1211-1218.

Dia, V.P. Bringe, N.A., and González de Mejía, E. (2014). Peptides in pepsin-pancreatin hydrolysates from commercially available soy products that inhibit lipopolysaccharide-induced inflammation in macrophages. *Food Chem*. **152**: 423-431.

Drago, S., El Asmar, R., Di Pierro, M., Grazia Clemente, M., Tripathi, A., Sapone, A., Thakar, M., Perezábad, G., Carroccio, A., D'Agate, C., Not, T., Zampini, L., Catassi, C., and Fasano, A. (2006). Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand. J. Gastroenterol*. **41**: 408-419.

Du Toit, G., Roberts, G., Sayre, P.H., Bahnson, H.T., Radulovic, S., Santos, A.F., Brough, H.A., Phippard, D., Basting, M., Feeney, M., Turcanu, V., Sever, M.L., Gomez Lorenzo, M., Plaut, M., and Lack, G.; LEAP Study Team. (2015). Randomized trial of peanut consumption in infants at risk for peanut allergy. *N. Engl. J. Med*. **372**: 803-813.

Duarte, J., Vinderola, G., Ritz, B., Perdigón, G., and Matar, C. (2006). Immunomodulating capacity of commercial fish protein hydrolysate for diet supplementation. *Immunobiology*. **211**: 341-350.

Durrieu, C., Degraeve, P., Chappaz, S., and Martial-Gros, A. (2006). Immunomodulating effects of water-soluble extracts of traditional French Alps cheeses on a human T-lymphocyte cell line *Int. Dairy J.* **16**: 1505-1514.

Easton, D.M., Nijnik, A., Mayer, M.L., and Hancock, R.E.W. (2009). Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol*. **27**: 582-590.

Eiwegger, T., Rigby, N., Mondoulet, L., Bernard, H., Krauth, M. T., Boehm, A., Dehlink, E., Valent, P., Wal, J.M., Mills, E.N.C., and Szépfalusi, Z. (2006). Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential. *Clin. Exp. Allergy*. **36**: 1281-1288.

Espeche Turbay, M.B., de Moreno de LeBlanc, A., Perdigón, G., Savoy de Giori, G., and Hebert, E.M. (2012). β-Casein hydrolysate generated by the cell envelope-associated proteinase of Lactobacillus delbrueckii ssp. lactis CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. *J. Dairy. Sci*. **95**: 1108-1118.

Fernández-Tomé, S., Ramos, S., Cordero-Herrera. I., Recio, I., Goya, L., and Hernández-Ledesma, B. (2014). In vitro chemo-protective effect of bioactive peptide lunasin against oxidative stress in human HepG2 cells. *Food Res. Int.* **62**: 793-800.

Finkelman, F.D. (2005). Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J. Allergy Clin. Immunol*. **115**: 449-457.

Finkelman, F.D. (2007). Anaphylaxis: lessons from mouse models. *J. Allergy Clin. Immunol*. **120**: 506-515.

Finkelman, F.D., Khodoun, M.V., and Strait, R. (2016). Human IgE-independent systemic anaphylaxis. *J. Allergy Clin. Immunol*. **137**: 1674-80.

Fitzgerald, A.J., Rai, P.S., Marchbank, T., Taylor, G.W., Ghosh, S., Ritz, B.W., and Playford, R.J. (2005). Reparative properties of a commercial fish protein hydrolysate preparation. *Gut*. **54**: 775-781.

Fritsché, R., Pahud, J.J., Pecquet, S., and Pfeifer, A. (1997). Induction of systemic immunologic tolerance to beta-lactoglobulin by oral administration of a whey protein hydrolysate. *J. Allergy Clin. Immunol*. **100**: 266-273.

Fritsché, R. (2009). Utility of animal models for evaluating hypoallergenicity. *Mol. Nutr. Food Res*. **53**: 979-983.

Galli, S.J, Tsai, M., and Piliponsky, A.M. (2008). The development of allergic inflammation. *Nature*. **454**: 445-454.

Galli, S.J., and Tsai, M. (2012). IgE and mast cells in allergic disease. *Nat. Med*. **18**: 693-704.

García-Alonso, M., Caballero, M.L., Umpierrez, A., Lluch-Bernal, M., Knaute, T., and Rodríguez-Pérez, R. (2015). Relationships between T cell and IgE/IgG4 epitopes of the Anisakis simplex major allergen Ani s 1. *Clin. Exp. Allergy*. **45**: 994-1005.

García-Nebot, M.J., Recio, I., and Hernández-Ledesma, B. (2014). Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells. *Food Chem. Toxicol*. **65**: 155-161.

García-Redondo, A. B., Roque, F. R., Miguel, M., López-Fandiño, R., and Salaices, M. (2010). Vascular effects of egg white-derived peptides in resistance arteries from rats. Structure-activity relationships. *J. Sci. Food Agric.* **90**: 1988-1993.

Gauthier, S. F., Pouliot, Y., and Saint-Sauveur, D. (2006). Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *Int. Dairy J.* **16**: 1315-1323.

Giavi, S., Vissers, Y.M., Muraro, A., Lauener, R., Konstantinopoulos, A.P., Mercenier, A., Wermeille, A., Lazzarotto, F., Frei, R., Bonaguro, R., Summermatter, S., Nutten, S., and Papadopoulos, N.G. (2016). Oral immunotherapy with low allergenic hydrolysed egg in egg allergic children. *Allergy*. doi: 10.1111/all.12905.

Giordani, L., Del Pinto, T., Vincentini, O., Felli, C., Silano, M., and Viora, M. (2014). Two wheat decapeptides prevent gliadin-dependent maturation of human dendritic cells. *Exp. Cell Res.* **321**: 248-254.

Gómez, E., Díaz-Perales, A., Tordesillas, L., Doña, I., Torres, M.J., Blázquez, A.B., Gómez, F., Blanca, M., and Mayorga, C. (2012). Effect of Pru p 3 on dendritic cell maturation and T-lymphocyte proliferation in peach allergic patients. *Ann. Allergy Asthma Immunol*. **109**: 52-58.

Gostner, J., Ciardi, C., Becker, K., Fuchs, D., and Sucher, R. (2014). Immunoregulatory Impact of Food Antioxidants. *Curr. Pharm. Des.***20**: 840-849.

Green, P.H.R., Lebwohl, B., and Greywoode, R. (2015). Celiac disease. . *J. Allergy Clin. Immunol.***135**:1099-1106.

Hacini-Rachinel, F., Vissers, Y.M., Doucet-Ladevéze, R., Blanchard, C., Demont, A., Perrot, M., Panchaud, A., Prioult, G., Mercenier, A., and Nutten, S. (2014). Low-allergenic hydrolyzed egg induces oral tolerance in mice. *Int. Arch. Allergy Immunol*. **164**: 64-73.

Haney, E.F., and Hancock, R.E. (2013). Peptide design for antimicrobial and immunomodulatory applications. *Biopolymers*. **100**: 572-583.

Hansen, S. E., Nielsen, K., and Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods*. **119**: 203-210.

Hasnat, M.A., Pervin, M., Kim, D.H., Kim, Y.J., Lee, J.J., Pyo, H.J., Lee, C.W., and Lim, B.O. (2015). DNA protection and antioxidant and anti-inflammatory activities of water extract and fermented hydrolysate of abalone (Haliotis discus hannai Ino). *J. Food Sci. Biotechnol.* **24**: 689-697.

He, X.Q., Cao, W.H., Pan, G.K., Yang, L., and Zhang, C.H. (2015). Enzymatic hydrolysis optimization of Paphia undulata and lymphocyte proliferation activity of the isolated peptide fractions. *J. Sci. Food Agric*. **95**: 1544-1553.

Hering, N.A., Andres, S., Fromm, A., van Tol, E.A., Amasheh, M., Mankertz, J., Fromm, M., and Schulzke, J.D. (2011). Transforming growth factor-β, a whey protein component, strengthens the intestinal barrier by upregulating claudin-4 in HT-29/B6 cells. *J. Nutr*. **141**: 783-789.

Hernández-Ledesma, B., Hsieh, C.C., and de Lumen, B.O. (2009). Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochem. Biophys. Res. Commun*. **390**: 803-808.

Heyman, M. (2005). Gut barrier dysfunction in food allergy. *Eur. J. Gastroenterol. Hepatol*. **17**: 1279-1285.

Hilchie, A. L., Wuerth, K., and Hancock, R .E. (2013). Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* **9**: 761-768.

Hirai, S., Horii, S., Matsuzaki, Y., Ono, S., Shimmura, Y., Sato, K., and Egashira, Y. (2014). Anti-inflammatory effect of pyroglutamyl-leucine on lipopolysaccharide-stimulated RAW 264.7 macrophages. *Life Sci*. **117**: 1-6.

Holen, E., Bolann, B., and Elsayed, S. (2001). Novel B and T cell epitopes of chicken ovomucoid (Gal d 1) induce T cell secretion of IL-6, IL-13, and IFN-gamma. *Clin. Exp. Allergy*. **31**: 952-964.

Hollon, J., Puppa, E.L., Greenwald, B., Goldberg, E., Guerrerio, A., and Fasano, A. (2015). Effect of gliadin on permeability of intestinal biopsy explants from celiac disease patients and patients with non-celiac gluten sensitivity. *Nutrients*. **7**: 1565-1576.

Holvoet, S., Zuercher, A.W., Julien-Javaux, F., Perrot, M., and Mercenier, A. (2013). Characterization of candidate anti-allergic probiotic strains in a model of Th2-skewed human peripheral blood mononuclear cells. *Int. Arch. Allergy Immunol.* **161**: 142-154.

Hou, H., Fan, Y., Li, B., Xue, C., and Yu, G. (2012). Preparation of immunomodulatory hydrolysates from Alaska pollock frame. *J. Sci. Food Agric*. **92**: 3029-3038.

Hsu, S.C., Chen, C.H., Tsai, S.H., Kawasaki, H., Hung, C.H., Chu, Y.T., Chang, H.W., Zhou, Y., Fu, J., Plunkett, B., Su, S.N., Vieths, S., Lee, R.T., Lee, Y.C., and Huang, S.K. (2010). Functional interaction of common allergens and a C-type lectin receptor, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), on human dendritic cells. *J. Biol. Chem.* **285**: 7903-7910.

Huang, W., Chakrabarti, S., Majumder, K., Jiang, Y., Davidge, S.T., and Wu, J. (2010). Egg-derived peptide IRW inhibits TNF-α-induced inflammatory response and oxidative stress in endothelial cells. *J. Agric. Food. Chem*. **58**: 10840-10846.

Hwang, J.W., Lee, S.J., Kim, Y.S., Kim, E.K., Ahn, C.B., Jeon, Y.J., Moon, S.H., Jeon, B.T., and Park, P.J. (2012). Purification and characterization of a novel peptide with inhibitory effects on colitis induced mice by dextran sulfate sodium from enzymatic hydrolysates of Crassostrea gigas. *Fish Shellfish Immunol*. **33**: 993-999.

Iacono, I., Tripodi, S., Calvani, M., Panetta, V., Verga, M.C., and Sopo, S.M. (2013). Specific oral tolerance induction with raw hen's egg in children with very severe egg allergy: a randomized controlled trial. *Pediatr. Allergy Immunol.* **24**: 66-74

Ikeda, K., Nakajima, H., Suzuki, K., Kagami, S., Hirose, K., Suto, A., Saito, Y., and Iwamoto, I. (2003). Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood*. **101**: 3594-3596.

Iliev, I.D., Mileti, E., Matteoli, G., Chieppa, M., and Rescigno, M. (2009). Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol*. **2**: 340-350.

Inoue, R., Matsushita, S., Kaneko, H., Shinoda, S., Sakaguchi, H., Nishimura, Y., and Kondo, N. (2001). Identification of beta-lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy. *Clin. Exp. Allergy*. **31**: 1126-1134.

Isobe, N., Suzuki, M., Oda, M., and Tanabe, S. (2008). Enzyme-modified cheese exerts inhibitory effects on allergen permeation in rats suffering from indomethacin-induced intestinal inflammation. *Biosci. Biotechnol. Biochem*. **72**: 1740-1745.

Järvinen, K.M., Beyer, K., Vila, L., Bardina, L., Mishoe, M., and Sampson, H.A. (2007). Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy*. **62**: 758-765.

Jelinkova, L., Tuckova, L., Cinova, J., Flegelova, Z., and Tlaskalova-Hogenova, H. (2004). Gliadin stimulates human monocytes to production of IL-8 and TNF-alpha through a mechanism involving NF-kappa B. *FEBS Lett.* **571**: 81-85.

Jiehui, Z.., Liuliu, M., Haihong, X., Yang, G., Yingkai, J., Lun, Z., Li, D. X., Dongsheng, Z., and Shaohui Z. (2014). Immunomodulating effects of casein-derived peptides QEPVL and QEPV on lymphocytes in vitro and in vivo. *Food Funct.* **5**: 2061-2069.

Jiménez-Saiz, R., Rupa, P., and Mine, Y. (2011). Immunomodulatory effects of heated ovomucoid-depleted egg white in a BALB/c mouse model of egg allergy. *J. Agric. Food Chem.* **59**: 13195-13202.

Jiménez-Saiz, R., López-Expósito, I., Molina, E., and López-Fandiño, R. (2013). IgE-binding and in vitro gastrointestinal digestibility of egg allergens in the presence of polysaccharides. *Food Hydrocol.* **30**: 597-605.

Jiménez-Saiz, R., Benedé, S., Miralles, B., López-Expósito, I., Molina, E., and López-Fandiño, R. (2014). Immunological behavior of in vitro digested egg-white lysozyme. *Mol. Nutr. Food Res.* **58**: 614-624.

Jongejan, L., and van Ree, R. (2014). Modified allergens and their potential to treat allergic disease. *Curr. Allergy Asthma Rep*. **14**: 478.

Jutel, M., Agache, I., Bonini, S., Burks, A.W., Calderon, M., Canonica, W., Cox, L., Demoly, P., Frew, A.J., O'Hehir, R., Kleine-Tebbe, J., Muraro, A., Lack, G., Larenas, D., Levin, M., Nelson, H., Pawankar, R., Pfaar, O., van Ree, R., Sampson, H., Santos, A.F., Du Toit, G., Werfel, T., Gerth van Wijk, R., Zhang, L., and Akdis, C.A. (2015). International consensus on allergy immunotherapy. *J. Allergy Clin. Immunol.* **136**: 556-568.

Kangsanant, S., Thongraun, C., Jansakul, C., Murkovic, M., and Seechamnanturakit, V. (2015). Purification and characterisation of antioxidant and nitric oxide inhibitory peptides from Tilapia (Oreochromis niloticus) protein hydrolysate. *Int. J. Food Sci. Tech*. **50**: 660-665.

Katayama, S., Xu, X., Fan, M.Z., and Mine, Y. (2006). Antioxidative stress activity of oligophosphopeptides derived from hen egg yolk phosvitin in Caco-2 cells. *J. Agric. Food. Chem*. **54**: 773-778.

Kawahara, T., Katayama, D., and Otani, H. (2004). Effect of beta-casein (1-28) on proliferative responses and secretory functions of human immunocompetent cell lines. *Biosci. Biotechnol. Biochem*. **68**: 2091-2095.

Kawahara, T., and Otani, H. (2004). Stimulatory effects of casein phosphopeptide (CPP-III) on mRNA expression of cytokines in Caco-2 cells. *Biosci. Biotechnol. Biochem*. **68**: 1779-1781.

Kean, D.E., Goodridge, H.S., McGuinness, S., Harnett, M.M., Alcocer, M.J., and Harnett, W. (2006). Differential polarization of immune responses by plant 2S seed albumins, Ber e 1, and SFA8. *J. Immunol*. **177**: 1561-1566.

Kiewiet, M.B., Gros, M., van Neerven, R.J., Faas, M.M., and de Vos, P. (2015). Immunomodulating properties of protein hydrolysates for application in cow's milk allergy. *Pediatr. Allergy Immunol*. **26**: 206-217

Kim, E.K., Kim, Y.S., Hwang, J.W., Kang, S.H., Choi, D.K., Lee, K.H., Lee, J.S., Moon, S.H., Jeon, B.T., and Park, P.J. (2013). Purification of a novel nitric oxide inhibitory peptide derived from enzymatic hydrolysates of Mytilus coruscus. *Fish Shellfish Immunol*. **34**:1416-1420.

Kitts, D.D., and Nakamura, S. (2006). Calcium-enriched casein phosphopeptide stimulates release of IL-6 cytokine in human epithelial intestinal cell line. *J. Dairy Res*. **73**: 44-48.

Knipping, K., van Esch, B.C., van Ieperen-van Dijk, A.G., van Hoffen, E., van Baalen, T., Knippels, L.M., van der Heide, S., Dubois, A.E., Garssen, J., and Knol, E.F. (2012). Enzymatic

treatment of whey proteins in cow's milk results in differential inhibition of IgE-mediated mast cell activation compared to T-cell activation. *Int. Arch. Allergy Immunol*. **159**: 263-270.

Ko, S.C., Lee, D.S., Park, W.S., Yoo, J.S., Yim, M.J., Qian, Z.J., Lee, C.M., Oh, J., Jung, W.K., and Choi, I.W. (2016). Anti-allergic effects of a nonameric peptide isolated from the intestine gastrointestinal digests of abalone (Haliotis discus hannai) in activated HMC-1 human mast cells. *Int. J. Mol. Med.* **37**: 243-250.

Kobayashi, Y., Kovacs-Nolan, J., Matsui, T., and Mine, Y. (2015). The Anti-atherosclerotic Dipeptide, Trp-His, Reduces Intestinal Inflammation through the Blockade of L-Type Ca 2+ Channels. *J. Agric. Food Chem.* **63**: 6041-6050.

Kondo, M., Kaneko, H., Fukao, T., Suzuki, K., Sakaguchi, H., Shinoda, S., Kato, Z., Matsui, E., Teramoto, T., Nakano, T., and Kondo, N. (2008). The response of bovine beta-lactoglobulin-specific T-cell clones to single amino acid substitution of T-cell core epitope. *Pediatr. Allergy Immunol*. **19**: 592-598.

Kong, X., Guo, M., Hua, Y., Cao, D., and Zhang, C. (2008). Enzymatic preparation of immunomodulating hydrolysates from soy proteins. *Bioresour. Technol*. **99**: 8873-8879.

Kong, J., Chalcraft, K., Mandur, T. S., Jimenez-Saiz, R., Walker, T. D., Goncharova, S., Gordon, M. E., Naji, L., Flader, K., Larché, M., Chu, D. K., Waserman, S., McCarry, B., and Jordana, M. (2015). Comprehensive metabolomics identifies the alarmin uric acid as a critical signal for the induction of peanut allergy. *Allergy*. **70**: 495-505.

Kotler, B.M., Kerstetter, J.E., and Insogna, K.L. (2013). Claudins, dietary milk proteins, and intestinal barrier regulation. *Nutr. Rev*. **71**: 60-65.

Kroghsbo, S., Andersen, N.B., Rasmussen, T.F., Jacobsen, S., and Madsen, C.B. (2014). Acid hydrolysis of wheat gluten induces formation of new epitopes but does not enhance sensitizing capacity by the oral route: a study in "gluten free" Brown Norway rats. *PLoS One*. **9**: e107137.

Kukkonen, K., Kuitunen, M., Haahtela, T., Korpela, R., Poussa, T., and Savilahti, E. (2010). High intestinal IgA associates with reduced risk of IgE-associated allergic diseases. *Pediatr. Allergy Immunol.* **21**: 67-73.

Kulis, M., Macqueen, I., Li, Y., Guo, R., Zhong, X.P., and Burks, A.W. (2012). Pepsinized cashew proteins are hypoallergenic and immunogenic and provide effective immunotherapy in mice with cashew allergy. *J. Allergy Clin. Immunol*. **130**: 716-723.

Ladics, G.S., Knippels, L.M., Penninks, A.H., Bannon, G.A., Goodman, R.E., and Herouet-Guicheney, C. (2010). Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. *Regul. Toxicol. Pharmacol*. **56**: 212-224.

Lahoute, C., Herbin, O., Mallat, Z., and Tedgui, A. (2011). Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nat. Rev. Cardiol*. **8**: 348-58.

Laparra, J.M., Alegría, A., Barberá, R., and Farré, R. (2008). Antioxidant effect of casein phosphopeptides compared with fruit beverages supplemented with skimmed milk against H2O2-induced oxidative stress in Caco-2 cells. *Food Res. Int*. **41**: 773-779.

Lawrence, T. (2009). The nuclear factor NF-κB pathway in inflammation. *Cold Spring Harb. Perspect. Biol*. **1**: a001651.

LeBlanc, J.G., Matar, C., Valdéz, J.C., LeBlanc, J., and Perdigon, G. (2002). Immunomodulating effects of peptidic fractions issued from milk fermented with Lactobacillus helveticus. *J Dairy Sci*. **85**: 2733-2742.

Lee, M., Kovacs-Nolan, J., Archbold, T., Fan, M.Z., Juneja, L.R,, Okubo, T., and Mine, Y. (2009). Therapeutic potential of hen egg white peptides for the treatment of intestinal inflammation. *J. Funct. Foods*. **1**: 1161-1169.

Lee, S.J., Kim, E.K., Kim, Y.S., Hwang, J.W., Lee, K.H., Choi, D.K., Kang, H., Moon, S.H., Jeon, B.T., and Park, P.J. (2012). Purification and characterization of a nitric oxide inhibitory peptide from Ruditapes philippinarum. *Food Chem. Toxicol*. **50**: 1660-1666.

Lee, J.B., Chen, C.Y., Liu, B., Mugge, L., Angkasekwinai, P., Facchinetti, V., Dong, C., Liu, Y.J., Rothenberg, M.E., Hogan, S.P., Finkelman, F.D., and Wang, Y.H. (2016). IL-25 and CD4(+) TH2 cells enhance type 2 innate lymphoid cell-derived IL-13 production, which promotes IgE-mediated experimental food allergy. *J. Allergy Clin. Immunol*. **137**: 1216-25.

Lenoir, M., Del Carmen, S., Cortes-Perez, N.G., Lozano-Ojalvo, D., Muñoz-Provencio, D., Chain, F., Langella, P., de Moreno de LeBlanc, A., LeBlanc, J.G., and Bermúdez-Humarán, L.G. (2016). Lactobacillus casei BL23 regulates Treg and Th17 T-cell populations and reduces DMH-associated colorectal cancer. *J Gastroenterol.*, **51**: 862-73.

Leonard, S.A., Martos, G., Wang, W., Nowak-Węgrzyn, A., and Berin, M.C. (2012). Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. *J. Allergy Clin. Immunol.* **129**: 1579-1587.

Li, J., Wang, Y., Tang, L., de Villiers W. J., Cohen, D., Woodward, J., Finkelman, F. D., and Eckhardt, E. R. (2013). Dietary medium-chain triglycerides promote oral allergic sensitization and orally induced anaphylaxis to peanut protein in mice. *J. Allergy Clin. Immunol.*, **131**: 442-450.

Lin, M.C., Lin, S.B., Lee, S.C., Lin, C.C., Hui, C.F., and Chen, J.Y. (2010). Antimicrobial peptide of an anti-lipopolysaccharide factor modulates of the inflammatory response in RAW264.7 cells. *Peptides*. **31**: 1262-1272.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. **25**: 402-8.

Lochner, M., Wang, A., and Sparwasser, T. (2015). The special relationship in the development and function of T helper 17 and regulatory T cells. *Prog. Mol. Biol. Transl. Sci.* **136:** 99-129.

López-Abarrategui, C., del Monte-Martinez, A., Reyes-Acosta, O., Franco, O.L., and Otero-González, A.J. (2013). LPS in mobilization on porous and non-porous supports as an approach for the isolation of anti-LPS host-defense peptides. *Front. Microbiol*. **4**: 389.

López-Expósito, I., Castillo, A., Yang, N., Liang, B., and Li, X. (2011). Chinese herbal extracts of *Rubia cordifolia* and *Dianthus superbus* suppress IgE production and prevent peanut-induced anaphylaxis. *Chin. Med.* **6**: 35.

López-Expósito, I., Chicón, R., Belloque, J., López-Fandiño, R., and Berin, M.C. (2012). In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of β-lactoglobulin are immunologically inert. *J. Dairy Sci*. **95**: 541-548.

López-Posadas, R., Requena, P., González, R., Suárez, M.D., Zarzuelo, A., Sánchez de Medina, F., and Martínez-Augustin, O. (2010). Bovine glycomacropeptide has intestinal antiinflammatory effects in rats with dextran sulfate-induced colitis. *J. Nutr*. **140**: 2014-9.

Lu, Y.C., Yeh, W.C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*. **42**: 145-151.

Macatonia, S.E., Hosken, N.A., Litton, M., Vieira, P., Hsieh, C.S., Culpepper, J.A., Wysocka, M., Trinchieri, G., Murphy, K.M., and O'Garra, A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J. Immunol.* **154**: 5071-5079.

MacDonald, T.T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science*. **307**: 1920-1925.

Mackenzie, K.J., Fitch, P.M., Leech, M.D., Ilchmann, A., Wilson, C., McFarlane, A.J., Howie, S.E., Anderton, S.M., and Schwarze, J. (2013). Combination peptide immunotherapy based on T-cell epitope mapping reduces allergen-specific IgE and eosinophilia in allergic airway inflammation. *Immunology*. **138**:258-268.

Malherbe, L. (2009). T-cell epitope mapping. *Ann. Allergy Asthma Immunol*. **103**: 76-79.

Malinowski, J., Klempt, M., Clawin-Rädecker, I., Lorenzen, P.C., and Meisel, H. (2014). Identification of a NFκB inhibitory peptide from tryptic β-casein hydrolysate. *Food Chem*. **165**: 129-133.

Mallegol, J., Van Niel, G., Lebreton, C., Lepelletier, Y., Candalh, C., Dugave, C., Heath, J.K., Raposo, G., Cerf-Bensussan, N., and Heyman, M. (2007). T84-intestinal epithelial exosomes bear MHC class II/peptide complexes potentiating antigen presentation by dendritic cells. *Gastroenterology*. **132**: 1866-1876.

Mallet, J.F., Duarte, J., Vinderola, G., Anguenot, R., Beaulieu, M., and Matar, C. (2014). The immunopotentiating effects of shark-derived protein hydrolysate. *Nutrition*. **30**: 706-712.

Manso, M. A., Miguel, M., Even, J., Hernández, R., Aleixandre, A. and López-Fandiño, R. (2008). Effect of the long-term intake of an egg white hydrolysate on the oxidative status and blood lipid profile of spontaneously hypertensive rats. *Food Chem.* **109**: 361-367.

Mao, X.Y., Yang, H.Y., Song, J.P., Li, Y.H., and Ren, F.Z. (2007). Effect of yak milk casein hydrolysate on TH1/TH2 cytokines production by murine spleen lymphocytes in vitro. *J. Agric. Food Chem.* **55**: 638-642.

Martínez-Augustin, O., Rivero-Gutiérrez, B., Mascaraque, C., and Sánchez de Medina, F. (2014). Food derived bioactive peptides and intestinal barrier function. *Int. J. Mol. Sci*. **15**: 22857-22873.

Martínez-Maqueda, D., Miralles, B., De Pascual-Teresa, S., Reverón, I., Muñoz, R., and Recio, I. (2012). Food-derived peptides stimulate mucin secretion and gene expression in intestinal cells. *J. Agric. Food Chem*. **60**: 8600-8605.

Martínez-Maqueda, D., Miralles, B., Cruz-Huerta, E., and Recio, I. (2013). Casein hydrolysate and derived peptides stimulate mucin secretion and gene expression in human intestinal cells. *Int. Dairy J.* **32**: 13-19.

Martínez-Villaluenga, C., Dia, V.P., Berhow, M., Bringe, N.A., and González de Mejía, E. (2009). Protein hydrolysates from beta-conglycinin enriched soybean genotypes inhibit lipid accumulation and inflammation in vitro. *Mol. Nutr. Food Res*. **53**: 1007-1018.

Martorell, A., Alonso, E., Boné, J., Echeverría, L., López, M. C., Martín, F., Nevot, S., and Plaza, A.M. (2013). Position document: IgE-mediated allergy to egg protein. *Allergol. Immunopathol.* **41**: 320-336.

Masilamani, M., Wei, J., Bhatt, S., Paul, M., Yakir, S., and Sampson, H.A. (2011). Soybean isoflavones regulate dendritic cell function and suppress allergic sensitization to peanut. *J. Allergy Clin. Immunol*. **128**: 1242-1250.

Masilamani, M., Wei, J., and Sampson, H. A. (2012). Regulation of the immune response by soybean isoflavones. Immunol. Res. **54**: 95-110.

Massoud, A.H., Charbonnier, L.M., Lopez, D., Pellegrini, M., Phipatanakul, W., and Chatila, T.A. (2016). An asthma-associated IL4R variant exacerbates airway inflammation by promoting conversion of regulatory T cells to TH17-like cells. *Nat. Med.* **22**: 1013-22.

McCarthy, A.L., O'Callaghan, Y.C., Connolly, A., Piggott, C.O., FitzGerald, R.J., and O'Brien, N.M. (2013). Brewers' spent grain (BSG) protein hydrolysates decrease hydrogen peroxide (H2O2)-induced oxidative stress and concanavalin-A (con-A) stimulated IFN-γ production in cell culture. *Food Funct.* **4**:1709-1716.

McCarthy, A.L., O'Callaghan, Y.C., Connolly, A., Piggott, C.O., FitzGerald, R.J., and O'Brien, N.M. (2016). A study of the ability of bioactive extracts from brewers' spent grain to enhance the antioxidant and immunomodulatory potential of food formulations following in vitro digestion. *Int. J. Food Sci. Nutr*. **66**: 230-235.

McDole, J.R., Wheeler, L.W., McDonald, K.G., Wang, B., Konjufca, V., Knoop, K.A., Newberry, R.D., and Miller, M.J. (2012). Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*. **483**: 345-349.

Meglio, P., Giampetro, P.G., Carello, R., Gabriele, I., Avitabile, S., and Galli, E. (2013). Oral food desensitization in children with IgE-mediated hen's egg allergy: a new protocol with raw hen's egg. *Pediatr. Allergy Immunol.* **24:** 75-83.

Ménard, S., Cerf-Bensussan, N., and Heyman, M. (2010). Multiple facets of intestinal permeability and epithelial handling of dietary antigens. *Mucosal Immunol*. **3**: 247-259.

Mercier, A., Gauthier, S.F., and Fliss, I. (2004). Immunomodulating effects of whey proteins and their enzymatic digests. *Int. Dairy J*. **14**: 175-183.

Mestas, J., and Hughes, C.C.W. (2004). Of mice and not men: Differences between mouse and human immunology. *J. Immunol*. **172**: 2731-2738.

Meulenbroek, L.A., van Esch, B.C., Hofman, G.A., den Hartog Jager, C.F., Nauta, A.J., Willemsen, L.E., Bruijnzeel-Koomen, C.A., Garssen, J., van Hoffen, E., and Knippels, L.M. (2013). Oral treatment with β-lactoglobulin peptides prevents clinical symptoms in a mouse model for cow's milk allergy. *Pediatr. Allergy Immunol*. **24**: 656-664.

Millán-Linares, M.D., Bermúdez, B., Yust, M.D., Millán, F., and Pedroche, J. (2014). Anti-inflammatory activity of lupine (Lupinus angustifolius L.) protein hydrolysates in THP-1-derived macrophages. *J. Funct. Foods* **8**: 224-233.

Mine, Y., Ma, F., and Lauriau, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *J. Agric. Food Chem.* **52**: 1088-1094.

Mine, Y., and Zhang, H. (2015). Anti-inflammatory Effects of Poly-l-lysine in Intestinal Mucosal System Mediated by Calcium-Sensing Receptor Activation. *J. Agric. Food Chem*. **63**: 10437-10447.

Miner-Williams, W.M., Stevens, B.R., and Moughan, P.J. (2014). Are intact peptides absorbed from the healthy gut in the adult human? *Nutr. Res. Rev.* **27**: 308-329.

Mochizuki, M., Shigemura, H., and Hasegawa, N. (2010). Anti-inflammatory effect of enzymatic hydrolysate of corn gluten in an experimental model of colitis. *J. Pharm. Pharmacol*. **62**: 389-392.

Moldaver, D., and Larché, M. (2011). Immunotherapy with peptides. *Allergy*. **66**: 784-791.

Montoya-Rodríguez, A., de Mejía, E.G., Dia, V.P., Reyes-Moreno, C., and Milán-Carrillo, J. (2014). Extrusion improved the anti-inflammatory effect of amaranth (Amaranthus hypochondriacus) hydrolysates in LPS-induced human THP-1 macrophage-like and mouse RAW 264.7 macrophages by preventing activation of NF-κB signaling. *Mol. Nutr. Food Res*. **58**: 1028-1041.

Morgan, A.J., Riley, L.G., Sheehy, P.A., and Wynn, P.C. (2014). The influence of protein fractions from bovine colostrum digested in vivo and in vitro on human intestinal epithelial cell proliferation. *J. Dairy. Res.* **81**: 73-81.

Muraro, A., Hoffmann-Sommergrube, K., Holzhauser, T., Poulsen, L. K., Gowland, M. H., Akdis, C. A., Mills, E. N., Papadopoulos, N., Roberts, G., Schnadt, S., van Ree, R., Sheikh, A., and Vieths, S. (2014). EAACI Food Allergy and Anaphylaxis Guidelines. Protecting consumers with food allergies: understanding food consumption, meeting regulations and identifying unmet needs, *Allergy*. **69**: 1464-1472.

Ndiaye, F., Vuong, T., Duarte, J., Aluko, R.E., and Matar, C. (2012). Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds. *Eur. J. Nutr*. **51**: 29-37.

Negishi, H., Miki, S., Sarashina, H., Taguchi-Atarashi, N., Nakajima, A., Matsuki, K., Endo, N., Yanai, H., Nishio, J., Honda, K., and Taniguchi, T. (2012). Essential contribution of IRF3 to intestinal homeostasis and microbiota-mediated TSLP gene induction. *Proc. Natl. Acad. Sci. U.S.A.*, **109**: 21016-21021.

Nelson, R., Katayama, S., Mine, Y., Duarte, J., and Matar, C. (2007). Immunomodulating effects of egg yolk low lipid peptic digests in a murine model. *Food Agric. Immunol*. **18**: 1-15.

Noval Rivas, M., Burton, O.T., Oettgen, H.C., and Chatila, T. (2016). IL-4 production by group 2 innate lymphoid cells promotes food allergy by blocking regulatory T-cell function. *J. Allergy Clin. Immunol*. **138**: 801-811.

Noval Rivas, M., and Chatila, T.A. (2016). Regulatory T cells in allergic diseases. *J. Allergy Clin. Immunol.* **138**: 639-52.

Nowak-Wegrzyn, A., and Albin, S. (2015). Oral immunotherapy for food allergy: mechanisms and role in management. *Clin. Exp. Allergy*. **45**: 368-383.

Nwaru, B., Hickstein, L., Panesar, S.S., Roberts, G., Muraro, A., and Sheikh, A. (2014). Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy*. **69**: 992-1007.

O'Hehir, R.E., Prickett, S.R., and Rolland, J.M. (2016). T cell epitope peptide therapy for allergic diseases. *Curr. Allergy Asthma Rep*. **16**: 14.

Ohnmacht, C., Park, J.H., Cording, S., Wing, J.B., Atarashi, K., Obata, Y., Gaboriau-Routhiau, V., Marques, R., Dulauroy, S., Fedoseeva, M., Busslinger, M., Cerf-Bensussan, N., Boneca, I.G., Voehringer, D., Hase, K., Honda, K., Sakaguchi, S., and Eberl, G. (2015). The microbiota regulates type 2 immunity through RORγt^{+} T cells. *Science*. **349**: 989-93.

Oseguera-Toledo, M.E., González de Mejía, E., Dia, V.P., and Amaya-Llano, S.L. (2011). Common bean (Phaseolus vulgaris L.) hydrolysates inhibit inflammation in LPS-induced macrophages through suppression of NF-κB pathways. *Food Chem.* **127**: 1175-1185.

Otani, H., Nakano, K., and Kawahara, T. (2003). Stimulatory effect of a dietary casein phosphopeptide preparation on the mucosal IgA response of mice to orally ingested lipopolysaccharide from Salmonella typhimurium. *Biosci. Biotechnol. Biochem.* **67**: 729-735.

Oyoshi, M.K., Oettgen, H.C., Chatila, T.A., Geha. R.S., and Bryce, P.J. (2014). Food allergy: Insights into etiology, prevention, and treatment provided by murine models. *J. Allergy Clin. Immunol*. **133**: 309-317.

Ozawa, T., Miyata, M., Nishimura, M., Ando, T., Ouyang, Y., Ohba, T., Shimokawa, N., Ohnuma, Y., Katoh, R., Ogawa, H., and Nakao, A. (2009). Transforming growth factor-beta activity in commercially available pasteurized cow milk provides protection against inflammation in mice. *J. Nutr*. **139**: 69-75.

Pablos-Tanarro, A., López-Expósito, I., Lozano-Ojalvo, D., López-Fandiño, R., and Molina, E. (2016). Antibody production, anaphylactic signs, and T-cell responses induced by oral sensitization with ovalbumin in BALB/c and C3H/HeOuJ mice. *Allergy Asthma Immunol. Res.* **8**: 239-245.

Palmer, D.J., Metcalfe, J., Makrides, M., Gold, M.S., Quinn, P., West, C.E., Loh, R., and Prescott, S.L. (2013). Early regular egg exposure in infants with eczema: a randomized controlled trial. *J. Allergy Clin. Immunol*. **132**: 387-392

Palomares, O., Yaman, G., Azkur, A.K., Akkoc, T., Akdis, M., and Akdis, C.A. (2010). Role of Treg in immune regulation of allergic diseases. *Eur. J. Immunol*. **40**: 1232-1240.

Palomares, O., Martín-Fontecha, M., Lauener, R., Traidl-Hoffmann, C., Cavkaytar, O., Akdis, M., and Akdis, C.A. (2014). Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. *Genes Immun*. **15**: 511-520.

Palova-Jelinkova, L., Rozkova, D., Pecharova, B., Bartova, J., Sediva, A., Tlaskalova-Hogenova, H., Spisek, R., and Tuckova, L. (2005). Gliadin fragments induce phenotypic and functional maturation of human dendritic cells. *J. Immunol*. **175**: 7038-7045.

Park, B.K., Park, S., Park, J.B., Park, M.C., Min, T.S., and Jin, M. (2013). Omega-3 fatty acids suppress Th2-associated cytokine gene expressions and GATA transcription factors in mast cells. *J. Nutr. Biochem.* **24**: 868-876.

Pascal, M., Konstantinou, G.N., Masilamani, M., Lieberman, J., and Sampson, H.A. (2013). In silico prediction of Ara h 2 T cell epitopes in peanut-allergic children. *Clin. Exp. Allergy*. **43**: 116-127.

Pastorello, E.A., Monza, M., Pravettoni, V., Longhi, R., Bonara, P., Scibilia, J., Primavesi, L., and Scorza, R. (2010). Characterization of the T-cell epitopes of the major peach allergen Pru p 3. *Int. Arch. Allergy Immunol.* **153**: 1-12.

Pecquet, S., Bovetto, L., Maynard, F., and Fritsché, R. (2000). Peptides obtained by tryptic hydrolysis of bovine beta-lactoglobulin induce specific oral tolerance in mice. *J. Allergy Clin. Immunol*. **105**: 514-521.

Pelaseyed, T., Bergström, J.H., Gustafsson, J.K., Ermund, A., Birchenough, G.M., Schütte, A., van der Post, S., Svensson, F., Rodríguez-Piñeiro, A.M., Nyström, E.E., Wising, C., Johansson, M.E., and Hansson, G.C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol. Rev*. **260**: 8-20.

Peng, H.J., Su, N.H., Tsai, J.J., Tsai, L.C., Kuo, H.L., and Kuo, S.W. (2004). Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naïve and sensitized mice. *Clin. Exp. Allergy*. **34**: 663-670.

Perezábad, L., Reche, M., Valbuena, T., López-Fandiño, R., Molina, E., and López-Expósito, I. (2015). Clinical efficacy and immunological changes subjacent to egg oral immunotherapy. *Ann. Allergy Asthma Immunol.* **114:** 504-509.

Perrier, C., and Corthésy, B. (2010). Gut permeability and food allergies. *Clin. Exp. Allergy*. **41**: 20-28.

Pesek, R.D., and Jones, S.M. (2016). Current and emerging therapies for IgE-mediated food allergy. *Curr. Allergy Asthma Rep.* **16**: 28.

Phelan, M., Aherne-Bruce, S.A., O'Sullivan, D.J., FitzGerald, R., and O'Brien, N.M. (2009). Potential bioactive effects of casein hydrolysates on human cultured cells. *Int. Dairy J*. **19:** 279-285.

Piccolomini, A.F., Iskandar, M.M., Lands, L.C., and Kubow, S. (2012). High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. *Food Nutr. Res*. **56**: 17549.

Plaisancié, P., Boutrou, R., Estienne, M., Henry, G., Jardin, J., Paquet, A., and Léonil, J. (2015). β-Casein (94-123)-derived peptides differently modulate production of mucins in intestinal goblet cells. *J. Dairy Res*. **82**: 36-46.

Platzer, B., Baker, K., Vera, M.P., Singer, K., Panduro, M., Lexmond, W.S., Turner, D., Vargas, S.O., Kinet, J.P., Maurer, D., Baron, R.M., Blumberg, R.S., and Fiebiger, E. (2015). Dendritic cell-bound IgE functions to restrain allergic inflammation at mucosal sites. *Mucosal Immunol.* **8**: 516-532.

Power, O., Jakeman, P., and FitzGerald, R.J. (2013). Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids.* **44**: 797-820.

Prickett, S.R., Voskamp, A.L., Dacumos-Hill, A., Symons, K., Rolland, J.M., and O'Hehir, R.E. (2011). Ara h 2 peptides containing dominant CD4+ T-cell epitopes: candidates for a peanut allergy therapeutic. *J. Allergy Clin. Immunol*. **127**: 608-615.

Prickett, S.R., Voskamp, A.L., Phan, T., Dacumos-Hill, A., Mannering, S.I., Rolland, J.M., and O'Hehir, R.E. (2013). Ara h 1 CD4+ T cell epitope-based peptides: candidates for a peanut allergy therapeutic. *Clin. Exp. Allergy*. **43**: 684-697.

Prickett, S.R., Rolland, J.M., and O'Hehir, R.E. (2015). Immunoregulatory T cell epitope peptides: the new frontier in allergy therapy. *Clin. Exp. Allergy*. **45**: 1015-1026.

Prioult, G., Pecquet, S., and Fliss, I. (2004). Stimulation of interleukin-10 production by acidic beta-lactoglobulin-derived peptides hydrolyzed with Lactobacillus paracasei NCC2461 peptidases. *Clin. Diagn. Lab. Immunol*. **11**: 266-271.

Prioult, G., Pecquet, S., and Fliss, I. (2005). Allergenicity of acidic peptides from bovine β-lactoglobulin is reduced by hydrolysis with Bifidobacterium lactis NCC362 enzymes. *Int. Dairy J.* **155**: 439-448.

Quah, B.J., and Parish, C.R. (2012). New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFSE-like fluorescent dyes. *J. Immunol. Methods*. **379**: 1-14.

Ravkov, E.V., Pavlov, I.Y., Martins, T.B., Gleich, G.J., Wagner, L.A., Hill, H.R., and Delgado, J.C. (2013). Identification and validation of shrimp-tropomyosin specific CD4 T cell epitopes. *Hum. Immunol*. **74**: 1542-1549.

Richard, N., Porath, D., Radspieler, A., and Schwager, J. (2005). Effects of resveratrol, piceatannol, tri-acetoxystilbene, and genistein on the inflammatory response of human peripheral blood leukocytes. *Mol. Nutr. Food Res.* **49**: 431-442.

Robertson, J.M., Jensen, P.E., and Evavold, B.D. (2000). DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J. Immunol.* **164**: 4706-4712.

Rodríguez-Carrio, J., Fernández, A., Riera, F.A., and Suárez, A. (2014). Immunomodulatory activities of whey β-lactoglobulin tryptic-digested fractions. *Int. Dairy J*. **34**: 65-73.

Rousset, F., Garcia, E., and Banchereau, J. (1991). Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med*. **173**: 705-710.

Ruiter, B., Trégoat, V., M'rabet, L., Garssen, J., Bruijnzeel-Koomen, C.A., Knol, E.F., and Hoffen, E. (2006). Characterization of T cell epitopes in alphas1-casein in cow's milk allergic, atopic and non-atopic children. *Clin. Exp. Allergy*. **36**: 303-310.

Ruiter V., and Shreffler, W.G. (2012). The role of dendritic cells in food allergy. *J. Allergy Clin. Immunol*. **129**: 921-928.

Rupa, P., and Mine, Y. (2012). Oral immunotherapy with immunodominant T-cell epitope peptides alleviates allergic reactions in a Balb/c mouse model of egg allergy. *Allergy*. **67**: 74-82.

Saigusa, M., Nishizawa, M., Shimizu, Y., and Saeki, H. (2015). In vitro and in vivo anti-inflammatory activity of digested peptides derived from salmon myofibrillar protein conjugated with a small quantity of alginate oligosaccharide. *Biosci. Biotechnol. Biochem.* **79**: 1518-1527.

Saint-Sauveur, D., Gauthier, S.F., Boutin, Y., and Montoni, A. (2008). Immunomodulating properties of a whey protein isolate, its enzymatic digest and peptide fractions. *Int. Dairy J.* **18**: 260-270.

Sallmann, E., Reininger, B., Brandt, S., Duschek, N., Hoflehner, E., Garner-Spitzer, E., Platzer, B., Dehlink, E., Hammer, M., Holcmann, M., Oettgen, H.C., Wiedermann, U., Sibilia, M., Fiebiger, E., Rot, A., and Maurer, D. (2011). High-affinity IgE receptors on dendritic cells exacerbate Th2-dependent inflammation. *J. Immunol*. **187**: 164-171.

Saluja, R., Ketelaar, M.E., Hawro, T., Church, M.K., Maurer, M., and Nawijn, M.C. (2015). The role of the IL-33/IL-1RL1 axis in mast cell and basophil activation in allergic disorders. *Mol. Immunol.* **63**: 80-85.

Samaranayaka, A.G.P., and Li-Chan, E.C.Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *J. Funct. Foods*. **3**: 229-254.

Sarlo, K., Kirchner, D.B., Troyano, E., Smith, L.A., Carr, G.J., and Rodriguez, C. (2010). Assessing the risk of type 1 allergy to enzymes present in laundry and cleaning products: evidence from the clinical data. *Toxicology*. **271**: 87-93.

Sarmadi, B.H., and Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides*. **31**: 1949-1956.

Scheurer, S., Toda, M., and Vieths, S. (2015). What makes an allergen? *Clin. Exp. Allergy*. **45**: 1483-1496.

Shan, M., Gentile, M., Yeiser, J.R., Walland, A.C., Bornstein, V.U., Chen, K., He, B., Cassis, L., Bigas, A., Cols, M., Comerma, L., Huang, B., Blander, J.M., Xiong, H., Mayer, L., Berin, C., Augenlicht, L.H., Velcich, A., and Cerutti, A. (2013). Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science*. **342**: 447-453.

Sharma, S., Chopra, K., Kulkarni, S.K., and Agrewala, J.N. (2007). Resveratrol and curcumin suppress immune response through CD28/CTLA-4 and CD80 co-stimulatory pathway. *Clin. Exp. Immunol.* **147**: 155-163.

Shin, J.S., and Greer, A.M. (2015). The role of FCεRI expressed in dendritic cells and monocites. *Cell. Mol. Life. Sci*. **72**: 2349-2360.

Shreffler, W.G., Castro, R.R., Kucuk, Z.Y., Charlop-Powers, Z., Grishina, G., Yoo, S., Burks, A.W., and Sampson, H.A. (2006). The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *J. Immunol*. **177**: 3677-3685.

Sicherer, S.H. (2011). Epidemiology of food allergy. *J Allergy Clin Immunol*. **127**: 594-602.

Sicherer, S.H., Wood, R.A., Vickery, B.P., Jones, S.M., Liu, A.H., Fleischer, D.M., Dawson, P., Mayer, L., Burks, A.W., Grishin, A., Stablein, D., and Sampson, H.A. (2014). The natural history of egg allergy in an observational cohort. *J. Allergy Clin. Immunol.* **133**: 492-499.

Singh, A., Holvoet, S., and Mercenier, A. (2011). Dietary polyphenols in the prevention and treatment of allergic diseases. *Clin. Exp. Allergy.* **41**: 1346-1359.

Soyer, O.U., Akdis, M., Ring, J., Behrendt, H., Crameri, R., Lauener, R., and Akdis, C.A. Mechanisms of peripheral tolerance to allergens. *Allergy*. **68**: 161-170.

Starkl, P., Krishnamurthy, D., Szalai, K., Felix, F., Lukschal, A., Oberthuer, D., Sampson, H.A., Swoboda, I., Betzel, C., Untersmayr, E., and Jensen-Jarolim, E. (2011). Heating affects structure, enterocyte adsorption and signalling, as well as immunogenicity of the peanut allergen Ara h 2. *Open Allergy J.* **4**: 24-34.

Strait, R.T., Morris, S.C., and Finkelman FD. (2006). IgG-blocking antibodies inhibit IgE-mediated anaphylaxis in vivo through both antigen interception and FcγRIIb cross-linking. *J. Clin. Invest.* **116**: 833-841.

Strait, R.T., Mahler, A., Hogan, S., Khodoun, M., Shibuya, A., and Finkelman FD. (2011). Ingested allergens must be absorbed systemically to induce systemic anaphylaxis. *J. Allergy Clin. Immunol.* **127**: 982-989.

Szajewska, H., and Horvath, A. (2010). Meta-analysis of the evidence for a partially hydrolyzed 100% whey formula for the prevention of allergic diseases. *Curr. Med. Res. Opin*. **26**: 423-437.

Tanabe, S., Isobe, N., Miyauchi, E., Kobayashi, S., Suzuki, M., and Oda, M. (2006). Identification of a peptide in enzymatic hydrolyzate of cheese that inhibits ovalbumin permeation in Caco-2 cells. *J. Agric. Food Chem*. **54**: 6904-6908.

Tanabe, S. (2012). Short peptide modules for enhancing intestinal barrier function. *Curr. Pharm. Des.* **18**: 776-781.

Thang, C.L., and Zhao, X. (2015). Effects of orally administered immunodominant T-cell epitope peptides on cow's milk protein allergy in a mouse model. *Food Res. Int*. **71**: 126-131.

Tordesillas, L., Cuesta-Herranz, J., González-Muñoz, M., Pacios, L.F., Compés, E., Garcia-Carrasco, B., Sanchez-Monge, R., Salcedo, G., and Diaz-Perales, A. (2009). T-cell epitopes of the major peach allergen, Pru p 3: Identification and differential T-cell response of peach-allergic and non-allergic subjects. *Mol. Immunol*. **46**: 722-728.

Tordesillas, L., Gómez-Casado, C., Garrido-Arandia, M., Murua-García, A., Palacín, A., Varela, J., Konieczna, P., Cuesta-Herranz, J., Akdis, C.A., O'Mahony, L., and Díaz-Perales, A. (2013). Transport of Pru p 3 across gastrointestinal epithelium - an essential step towards the induction of food allergy? *Clin. Exp. Allergy*. **43**: 1374-1383.

Trompette, A., Claustre, J., Caillon, F., Jourdan, G., Chayvialle, J.A., and Plaisancié, P. (2003). Milk bioactive peptides and beta-casomorphins induce mucus release in rat jejunum. *J. Nutr*. **133**: 3499-3503.

Tu, Y., Salim, S., Bourgeois, J., Di Leo, V., Irvine, E.J., Marshall, J.K., and Perdue, M.H. (2005). CD23-Mediated IgE transport across human intestinal epithelium: inhibition by blocking sites of translation or binding. *Gastroenterology*. **129**: 928-940.

Tuckova, L., Novotna, J., Novak, P., Flegelova, Z., Kveton, T., Jelinkova, L., Zidek, Z., Man, P., and Tlaskalova-Hogenova, H. (2002). Activation of macrophages by gliadin fragments: isolation and characterization of active peptide. *J. Leukoc. Biol.* **71**: 625-631.

Udenigwe, C.C., Je, J.Y., Cho, Y.S., and Yada, R.Y. (2013). Almond protein hydrolysate fraction modulates the expression of proinflammatory cytokines and enzymes in activated macrophages. *Food Funct.* **4**: 777-783.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., and Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *J. Nutr.* **141**: 769-776.

Untersmayr, E., Vestergaard, H., Malling, H.J., Jensen, L.B., Platzer, M.H., Boltz-Nitulescu, G, Scheiner, O., Skov, P.S., Jensen-Jarolim, E., and Poulsen, L.K. (2007). Incomplete digestion of codfish represents a risk factor for anaphylaxis in patients with allergy. *J. Allergy Clin. Immunol.* **119**: 711-717.

Utsch, L., Folisi, C., Akkerdaas, J.H., Logiantara, A., van de Pol, M.A., van der Zee, J.S., Krop, E.J., Lutter, R., van Ree, R., and van Rijt, L.S. (2015). Allergic sensitization is associated with inadequate antioxidant responses in mice and men. *Allergy*. **70**: 1246-1258.

Valenta, R., Campana, R., Focke-Tejkl, M., and Niederberger, V. (2016). Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: Lessons from the past and novel mechanisms of action for the future. *J. Allergy Clin. Immunol*. **137**: 351-357.

van de Veen, W., Stanic, B., Yaman, G., Wawrzyniak, M., Söllner, S., Akdis, D.G., Rückert, B., Akdis, C.A., and Akdis, (2013). M. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J. Allergy Clin. Immunol*. **131**:1204-1212.

van den Elsen, L.W., van Esch, B.C., Hofman, G.A., Kant, J., van de Heijning, B.J., Garssen, J., and Willemsen L.E. (2013a). Dietary long chain n-3 polyunsaturated fatty acids prevent allergic sensitization to cow's milk protein in mice. *Clin. Exp. Allergy*. **43**: 798-810.

van den Elsen, L.W., Nusse, Y., Balvers, M., Redegeld, F.A., Knol, E.F., Garssen, J., and Willemsen, L.E. (2013b). n-3 Long-chain PUFA reduce allergy-related mediator release by human mast cells in vitro via inhibition of reactive oxygen species. *Br. J. Nutr.* **109**: 1821-1831.

van den Elsen, L.W., van Esch, B.C., Dingjan, G.M., Hofman, G.A., Garssen, J., and Willemsen, L.E. (2015). Increased intake of vegetable oil rich in n-6 PUFA enhances allergic symptoms and prevents oral tolerance induction in whey-allergic mice. *Br. J. Nutr.* **114**: 577-585.

van Esch, B.C., Schouten, B., Hofman, G.A., van Baalen, T., Nijkamp, F.P., Knippels, L.M., Willemsen, L.E., and Garssen, J. (2010). Acute allergic skin response as a new tool to evaluate the allergenicity of whey hydrolysates in a mouse model of orally induced cow's milk allergy. *Pediatr. Allergy Immunol*. **21**: 780-786.

van Esch, B.C., Knipping, K., Jeurink, P., van der Heide, S., Dubois, A.E., Willemsen. L.E., Garssen, J., and Knippels, L.M. (2011a). In vivo and in vitro evaluation of the residual allergenicity of partially hydrolysed infant formulas. *Toxicol. Lett.* **201**: 264-269.

van Esch, B.C., Schouten, B., de Kivit, S., Hofman, G.A., Knippels, L.M., Willemsen, L.E., and Garssen, J. (2011b). Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3+ regulatory T-cells in the mesenteric lymph nodes. *Pediatr. Allergy Immunol.* **22**: 820-826.

van Esch, B.C., van Bilsen, J.H., Jeurink, P.V., Garssen, J., Penninks, A.H., Smit, J.J., Pieters, R,H., and Knippels, L.M. (2013). Interlaboratory evaluation of a cow's milk allergy mouse model to assess the allergenicity of hydrolysed cow's milk based infant formulas. *Toxicol. Lett.* **220**: 95-102.

Van Gramberg, J.L., de Veer, M.J., O'Hehir, R.E., Meeusen, E.N., and Bischof, R.J. (2013). Use of animal models to investigate major allergens associated with food allergy. *J. Allergy*. **2013**: 635695.

Van Niel, G., Raposo, G., Candalh, C., Boussac, M., Hershberg, R., Cerf-Bensussan, N., and Heyman, M. (2001). Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology*. **121**: 337-349.

Van Niel, G., Mallegol, J., Bevilacqua, C., Candalh, C., Brugière, S., Tomaskovic-Crook, E., Heath, J.K., Cerf-Bensussan, N., and Heyman, M. (2003). Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut*. **52**: 1690-1697.

Vandenplas, Y., Bhatia, J., Shamir, R., Agostoni, C., Turck, D., Staiano, A., and Szajewska, H. (2014). Hydrolyzed formulas for allergy prevention. *J. Pediatr. Gastroenterol. Nutr*. **58**: 549-552.

Vázquez-Ortiz, M., and Turner, P.J. (2016). Improving the safety of oral immunotherapy for food allergy. *Pediatr. Allergy Immunol*. 27: 117-125.

Veldhoen, M., Hocking, R., Atkins, C., Locksley, R.M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. **24**: 179-189.

Vernaza, M.G., Dia, V.P., de Mejía, E.G., and Chang, Y.K. (2012). Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chem*. **134**: 2217-2225.

Vickery, B.P. (2012). Egg oral immunotherapy. *Curr. Opin. Allergy Clin. Immunol*. **12**: 278-282.

Vinderola, C. G., Duarte, J., Thangavel, D., Perdigón, G., Farnworth, E., and Matar, C. (2005). Immunomodulating capacity of kefir, *J. Dairy Res*. **72**: 195-202.

Visser, J.T., Lammers, K., Hoogendijk, A., Boer, M.W., Brugman, S., Beijer-Liefers, S., Zandvoort, A., Harmsen, H., Welling, G., Stellaard, F., Bos, N.A., Fasano, A., and Rozing, J. (2010). Restoration of impaired intestinal barrier function by the hydrolysed casein diet contributes to the prevention of type 1 diabetes in the diabetes-prone BioBreeding rat. *Diabetologia*. **53**: 2621-2628.

Visser, J.T.J., Bos, N.A., Harthoorn, L.F., Stellaard, F., Beijer-Liefers, S., Rozing, J., and van Tol, E.A.F. (2012). Potential mechanisms explaining why hydrolyzed casein-based diets outclass single amino acid-based diets in the prevention of autoimmune diabetes in diabetes-prone BB rats. *Diabetes Metab. Res. Rev*. **28**: 505-513.

Vo, T.S., Ryu, B., and Kim, S.K. (2013). Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal Spirulina maxima. *J. Funct. Foods*. **5**: 1336-1346. ·

Wada, S., Sato, K., Ohta, R., Wada, E., Bou, Y., Fujiwara, M., Kiyono, T., Park, E.Y., Aoi, W., Takagi, T., Naito, Y., and Yoshikawa, T. (2013). Ingestion of low dose pyroglutamyl leucine improves dextran sulfate sodium-induced colitis and intestinal microbiota in mice. *J. Agric. Food Chem*. **61**: 8807-8813.

Wai, C.Y., Leung, N.Y., Ho, M.H., Gershwin, L.J., Shu, S.A., Leung, P.S., and Chu, K.H. (2014). Immunization with Hypoallergens of shrimp allergen tropomyosin inhibits shrimp tropomyosin specific IgE reactivity. *PLoS One*. **9**:e111649.

Wai, C.Y., Leung, N.Y., Leung, P.S., and Chu, K.H. (2016). T cell epitope immunotherapy ameliorates allergic responses in a murine model of shrimp allergy. *Clin. Exp. Allergy*. **46**: 491-503.

Wang, S.P., Delgado, J.C., Ravkov, E., Eckels, D.D., Georgelas, A., Pavlov, I.Y., Cusick, M., Sebastian, K., Gleich, G.J., and Wagner, L.A. (2012). Penaeus monodon tropomyosin induces CD4 T-cell proliferation in shrimp-allergic patients. *Hum. Immunol*. **73**: 426-431.

Wood, R.A. (2016). Food allergen immunotherapy: Current status and prospects for the future. *J. Allergy Clin. Immunol.* **137**: 973-982.

Worthington, J.J. (2015). The intestinal immunoendocrine axis: Novel cross-talk between enteroendocrine cells and the immune system during infection and inflammatory disease. *Biochem. Soc. Trans*. **43**: 727-733.

Xu, H., Liew, L.N., Kuo, I.C., Huang, C.H., Goh, D.L., and Chua, K.Y. (2008). The modulatory effects of lipopolysaccharide-stimulated B cells on differential T-cell polarization. *Immunology*. **125**: 218-228.

Xu, C., Yang, C., Yin, Y., Liu, J., and Mine, Y. (2012). Phosphopeptides (PPPs) from hen egg yolk phosvitin exert anti-inflammatory activity via modulation of cytokine expression. *J. Funct. Foods*. **4**: 718–726.

Yang, M., and Mine, Y. (2009). Novel T-cell epitopes of ovalbumin in BALB/c mouse: Potential for peptide-immunotherapy. *Biochem. Biophys. Res. Commun.* **378**: 203-208.

Yang, R., Zhang, Z., Pei, X., Han, X., Wang, J., Wang, L., Long, Z., Shen, X., and Li, Y. (2009a). Immunomodulatory effects of marine oligopeptide preparation from Chum Salmon (Oncorhynchus keta) in mice. *Food Chem*. **113**: 464-470.

Yang, M., Yang, C., Nau, F., Pasco, M., Juneja, L. R., Okubo T., and Mine, Y. (2009b). Immunomodulatory effects of egg white enzymatic hydrolysates containing immunodominant epitopes in a BALB/c mouse model of egg allergy. *J. Agric. Food Chem.* **57**: 2241-2248.

Yang, M., Yang, C., and Mine, Y. (2010). Multiple T cell epitope peptides suppress allergic responses in an egg allergy mouse model by the elicitation of forkhead box transcription factor 3- and transforming growth factor-β-associated mechanisms. *Clin. Exp. Allergy*. **40**: 668-678.

Yano, S., Umeda, D., Yamashita, T., Ninomiya, Y., Sumida, M., Fujimura, Y., Yamada, K., Tachibana, H. (2007). Dietary flavones suppresses IgE and Th2 cytokines in OVA-immunized BALB/c mice. *Eur. J. Nutr*. **46**: 257-263.

Yasumatsu, H., and Tanabe, S. (2010). The casein peptide Asn-Pro-Trp-Asp-Gln enforces the intestinal tight junction partly by increasing occludin expression in Caco-2 cells. *Br. J. Nutr*. **104**: 951-956.

You, S.J., Udenigwe, C.C., Aluko, R.E., and Wu, J. (2010). Multifunctional peptides from egg white lysozyme. *Food Res. Int.* **43**: 848-855.

Yu, Z., Yin, Y., Zhao, W., Chen, F., and Liu, J. (2014). Application and bioactive properties of proteins and peptides derived from hen eggs: opportunities and challenges. *J. Sci. Food Agric.* **94:** 2839-4285.

Yu, W., Hussey Freeland, D.M., and Nadeau, K.C. (2016). Food allergy: immune mechanisms, diagnosis and immunotherapy. *Nat. Rev. Immunol.* doi:10.1038/nri.2016.111

Zaknun, D., Schroecksnadel, S., Kurz, K., Fuchs, D. (2012). Potential role of antioxidant food supplements, preservatives and colorants in the pathogenesis of allergy and asthma. *Int. Arch. Allergy Immunol.* **157**: 113-124.

Zhang, C., Gui, L., Xu, Y., Wu, T., and Liu, D. (2013). Preventive effects of andrographolide on the development of diabetes in autoimmune diabetic NOD mice by inducing immune tolerance. *Int. Immunopharmacol.,* **16**: 451-6.

Zhang, H., Kovacs-Nolan, J., Kodera, T., Eto, Y., and Mine, Y. (2015). γ-Glutamyl cysteine and γ-glutamyl valine inhibit TNF-α signaling in intestinal epithelial cells and reduce inflammation in a mouse model of colitis via allosteric activation of the calcium-sensing receptor. *Biochim. Biophys. Acta*. **1852**: 792-804.

Zhao, A., Urban, J.F., Sun, R., Stiltz, J., Morimoto, M., Notari, L., Madden, K.B., Yang, Z., Grinchuk, V., Ramalingam, T.R., Wynn, T.A., and Shea-Donohue, T. (2010). Critical role of IL-25 in nematode infection-induced alterations in intestinal function. *J. Immunol*. **185**: 6921-9.

Zhou, J.S., Sandomenico, A., Severino, V., Burton, O.T., Darling, A., Oettgen, H.C., and Ruvo, M. (2013). An IgE receptor mimetic peptide (PepE) protects mice from IgE mediated anaphylaxis. *Mol. Biosyst*. **9**: 2853-2859.

Ziegler, S.F., and Artis D. (2010). Sensing the outside world: TSLP regulates barrier immunity *Nat. Immunol*. **11**: 289-293.

Zoghbi, S., Trompette, A., Claustre, J., El Homsi, M., Garzón, J., Jourdan, G., Scoazec, J.Y., and Plaisancié, P. (2006). Beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a mu-opioid pathway. *Am. J. Physiol. Gastrointest. Liver Physiol*. **290**: G1105-1113.

Zolkipli, Z., Roberts, G., Cornelius, V., Clayton, B., Pearson, S., Michaelis, L., Djukanovic, R., Kurukulaaratchy, R., and Arshad, S.H. (2015). Randomized controlled trial of primary prevention of atopy using house dust mite allergen oral immunotherapy in early childhood. *J. Allergy Clin. Immunol.* **136**: 1541-1547.

Zuercher, A.W., Holvoet, S., Weiss, M., Mercenier, A. (2010). Polyphenol-enriched apple extract attenuates food allergy in mice. *Clin. Exp. Allergy.* **40**: 942-950.

7. ANNEXES

Annexe I. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the hydrolysate of ovalbumin with pepsin.

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1204.6	1204.6	1+	23	20.8	17 - 26	KELKVHHANE
947.5	947.5	1+	22	20.5	19 - 26	LKVHHANE
834.4	834.4	1+	16	16.0	20 - 26	KVHHANE
566.2	566.3	1+	13	31.5	30 - 34	YCPIA
522.2	522.3	1+	19	30.4	42 - 46	VYLGA
765.4	765.4	1+	8	26.5	42 - 48	VYLGAKD
953.5	953.5	1+	45	26.3	42 - 50	VYLGAKDST
1210.6	1210.6	1+	15	26.0	42 - 52	VYLGAKDSTRT
783.4	1565.8	2+	11	29.2	42 - 55	VYLGAKDSTRTQIN
948.5	948.5	1+	9	19.7	44 - 52	LGAKDSTRT
660.0	1318.7	2+	16	19.9	45 - 56	GAKDSTRTQINK
707.3	707.4	1+	8	13.9	47 - 52	KDSTRT
948.4	948.5	1+	12	23.4	47 - 54	KDSTRTQI
1062.6	1062.6	1+	7	20.2	47 - 55	KDSTRTQIN
676.4	676.4	1+	10	37.5	61 - 66	DKLPGF
848.4	848.4	1+	25	34.8	61 - 68	DKLPGFGD
566.1	566.2	1+	15	13.9	72 - 77	AQCGTS
665.3	665.3	1+	10	21.8	72 - 78	AQCGTSV
779.3	779.3	1+	6	19.0	72 - 79	AQCGTSVN
878.4	878.4	1+	7	27.3	72 - 80	AQCGTSVNV
755.4	755.4	1+	21	28.6	78 - 84	VNVHSSL
656.3	656.3	1+	21	25.0	79 - 84	NVHSSL
758.4	758.4	1+	6	28.3	85 - 90	RDILNQ
929.5	929.5	1+	13	20.9	89 - 96	NQITKPND
1028.5	1028.5	1+	12	25.6	89 - 97	NQITKPNDV
687.3	687.4	1+	17	19.7	91 - 96	ITKPND
949.5	949.5	1+	18	28.1	91 - 98	ITKPNDVY
1036.5	1036.5	1+	12	26.1	91 - 99	ITKPNDVYS
533.1	533.3	1+	16	22.8	101 - 105	SLASR
646.4	646.4	1+	5	32.9	101 - 106	SLASRL
667.2	667.3	1+	11	21.1	107 - 111	YAEER
830.4	830.4	1+	21	24.4	107 - 112	YAEERY
790.4	790.4	1+	19	38.9	110 - 115	ERYPIL
719.4	719.4	1+	20	24.7	119 - 124	LQCVKE
832.4	832.5	1+	43	34.8	119 - 125	LQCVKEL
807.4	807.4	1+	6	29.2	125 - 131	LYRGGLE
694.4	694.4	1+	7	24.8	126 - 131	YRGGLE
1018.5	1018.5	1+	5	32.0	126 - 134	YRGGLEPIN
1165.6	1165.6	1+	12	42.3	126 - 135	YRGGLEPINF
633.2	633.3	1+	18	13.8	136 - 141	QTAADQ
704.2	704.3	1+	10	16.8	136 - 142	QTAADQA
731.4	731.4	1+	6	26.7	143 - 148	RELINS
747.3	747.4	1+	9	29.8	146 - 151	INSWVE
749.3	749.3	1+	11	25.2	149 - 154	WVESQT
920.4	920.4	1+	5	24.3	149 - 156	WVESQTNG
734.3	734.3	1+	12	14.9	150 - 156	VESQTNG
506.0	506.2	1+	17	7.5	152 - 156	SQTNG
727.5	727.5	1+	17	36.0	157 - 162	IIRNVL
1126.6	1126.7	1+	9	33.6	157 - 166	IIRNVLQPSS
630.3	630.3	1+	28	21.5	161 - 166	VLQPSS
729.4	729.4	1+	16	28.8	161 - 167	VLQPSSV
844.4	844.4	1+	11	26.7	161 - 168	VLQPSSVD
1059.5	1059.5	1+	11	25.0	161 - 170	VLQPSSVDSQ
632.3	632.3	1+	7	20.4	163 - 168	QPSSVD
719.2	719.3	1+	17	17.0	164 - 170	PSSVDSQ

652.2	652.3	1+	10	20.1	168 - 173	DSQTAM
537.2	537.2	1+	9	20.0	169 - 173	SQTAM
515.2	515.3	1+	11	27.6	174 - 178	VLVNA
953.4	953.4	1+	13	21.8	189 - 196	FKDEDTQA
806.3	806.4	1+	18	16.0	190 - 196	KDEDTQA
593.3	593.3	1+	14	42.5	195 - 199	QAMPF
879.4	879.4	1+	13	35.6	197 - 203	MPFRVTE
1007.5	1007.5	1+	7	34.1	197 - 204	MPFRVTEQ
748.4	748.4	1+	19	30.1	198 - 203	PFRVTE
876.4	876.5	1+	8	28.6	198 - 204	PFRVTEQ
779.4	779.4	1+	13	26.6	199 - 204	FRVTEQ
815.4	815.4	1+	10	17.8	204 - 210	QESKPVQ
946.5	946.5	1+	5	25.5	204 - 211	QESKPVQM
687.3	687.4	1+	16	17.4	205 - 210	ESKPVQ
818.4	818.4	1+	15	25.3	205 - 211	ESKPVQM
949.4	949.4	1+	19	30.6	205 - 212	ESKPVQMM
593.3	593.3	1+	22	38.6	213 - 217	YQIGL
710.3	710.4	1+	5	34.5	218 - 223	FRVASM
565.2	565.3	1+	9	19.5	224 - 228	ASEKM
806.4	806.4	1+	19	28.6	224 - 230	ASEKMKI
1048.6	1048.6	1+	13	33.2	224 - 232	ASEKMKILE
761.5	761.5	1+	7	32.3	227 - 232	KMKILE
874.6	874.5	1+	14	41.9	227 - 233	KMKILEL
615.4	615.4	1+	16	39.4	229 - 233	KILEL
823.4	823.4	1+	14	39.6	233 - 240	LPFASGTM
579.3	579.3	1+	20	23.5	234 - 239	PFASGT
797.3	797.3	1+	20	28.4	234 - 241	PFASGTMS
685.3	685.4	1+	19	34.1	244 - 249	VLLPDE
829.3	829.4	1+	13	37.3	246 - 253	LPDEVSGL
722.3	722.4	1+	13	42.6	257 - 262	ESIINF
619.3	619.3	1+	18	21.5	263 - 267	EKLTE
822.4	822.4	1+	5	41.3	267 - 273	EWTSSNV
594.2	594.3	1+	8	19.2	268 - 272	WTSSN
901.5	901.5	1+	16	25.9	275 - 281	EERKIKV
1177.7	1177.7	1+	11	36.8	275 - 283	EERKIKVYL
935.6	935.6	1+	11	27.7	276 - 282	ERKIKVY
1048.6	1048.7	1+	7	36.4	276 - 283	ERKIKVYL
662.3	662.3	1+	13	26.3	284 - 288	PRMKM
725.3	725.3	1+	6	29.8	307 - 313	FSSSANL
374.9	748.4	2+	17	26.5	309 - 316	SSANLSGI
521.1	521.3	1+	12	20.7	314 - 319	SGISSA
1132.6	1132.6	1+	16	23.8	323 - 333	KISQAVHAAHA
605.4	605.3	1+	6	16.2	327 - 332	AVHAAH
676.3	676.4	1+	26	18.6	327 - 333	AVHAAHA
805.4	805.4	1+	20	18.4	327 - 334	AVHAAHAE
918.5	918.5	1+	46	27.2	327 - 335	AVHAAHAEI
1161.6	1161.6	1+	23	24.2	327 - 337	AVHAAHAEINE
605.3	605.3	1+	11	17.6	328 - 333	VHAAHA
632.3	632.3	1+	11	16.5	334 - 339	EINEAG
675.3	675.3	1+	11	15.3	336 - 341	NEAGRE
687.4	687.4	1+	7	21.9	338 - 344	AGREVVG
845.4	845.4	1+	22	22.6	338 - 346	AGREVVGSA
432.1	432.2	1+	19	18.1	342 - 346	VVGSA
632.3	632.3	1+	6	20.3	342 - 348	VVGSAEA
790.4	790.4	1+	14	25.1	346 - 354	AEAGVDAAS
550.2	550.2	1+	17	17.0	354 - 358	SVSEE
1131.6	1131.6	1+	7	40.5	358 - 366	EFRADHPFL
1002.5	1002.5	1+	11	39.9	359 - 366	FRADHPFL
742.4	742.4	1+	10	27.9	360 - 365	RADHPF
855.5	855.4	1+	18	36.4	360 - 366	RADHPFL

1046.5	1046.5	1+	21	32.6	367 - 375	FCIKHIATN
785.4	785.4	1+	8	24.5	368 - 374	CIKHIAT
899.5	899.5	1+	13	22.7	368 - 375	CIKHIATN
970.5	970.5	1+	12	24.5	368 - 376	CIKHIATNA
682.4	682.4	1+	12	23.4	369 - 374	IKHIAT
796.4	796.5	1+	16	21.3	369 - 375	IKHIATN
701.4	701.4	1+	9	42.2	372 - 378	IATNAVL
629.3	629.3	1+	5	33.6	379 - 383	FFGRC

Annexe II. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 1 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP1).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
835.5	835.4	1+	15	15.6	45 - 52	GAKDSTRT
734.4	734.3	1+	13	15.0	150 - 156	VESQTNG
806.4	806.4	1+	33	15.9	190 - 196	KDEDTQA
687.5	687.4	1+	16	17.3	205 - 210	ESKPVQ
619.4	619.3	1+	15	21.3	263 - 267	EKLTE
594.3	594.3	1+	16	19.1	268 - 272	WTSSN
676.4	676.4	1+	16	18.3	327 - 333	AVHAAHA
805.4	805.4	1+	25	18.1	327 - 334	AVHAAHAE
692.3	692.3	1+	23	18.9	352 - 358	AASVSEE

Annexe III. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 2 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP2).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1204.8	1204.6	1+	37	20.4	17 - 26	KELKVHHANE
947.6	947.5	1+	38	20.3	19 - 26	LKVHHANE
522.3	522.3	1+	17	30.0	42 - 46	VYLGA
765.5	765.4	1+	16	26.1	42 - 48	VYLGAKD
953.6	953.5	1+	35	25.9	42 - 50	VYLGAKDST
605.9	1210.6	2+	8	25.6	42 - 52	VYLGAKDSTRT
1190.7	1190.6	1+	7	20.2	45 - 55	GAKDSTRTQIN
1133.7	1133.6	1+	11	20.0	46 - 55	AKDSTRTQIN
858.6	858.5	1+	21	23.3	51 - 57	RTQINKV
848.6	848.4	1+	19	34.5	61 - 68	DKLPGFGD
755.5	755.4	1+	18	28.2	78 - 84	VNVHSSL
656.4	656.3	1+	23	24.6	79 - 84	NVHSSL
758.5	758.4	1+	13	27.9	85 - 90	RDILNQ
929.6	929.5	1+	28	20.6	89 - 96	NQITKPND
687.4	687.4	1+	19	19.4	91 - 96	ITKPND
786.5	786.4	1+	18	24.1	91 - 97	ITKPNDV
830.5	830.4	1+	21	24.0	107 - 112	YAEERY
790.5	790.4	1+	14	38.8	110 - 115	ERYPIL
887.6	887.5	1+	27	37.0	111 - 117	RYPILPE
904.6	904.5	1+	15	35.9	126 - 133	YRGGLEPI
731.4	731.4	1+	8	26.3	143 - 148	RELINS
920.5	920.4	1+	22	23.8	149 - 156	WVESQTNG
727.5	727.5	1+	7	35.8	157 - 162	IIRNVL
729.5	729.4	1+	14	28.4	161 - 167	VLQPSSV
931.6	931.5	1+	14	25.7	161 - 169	VLQPSSVDS
1059.6	1059.5	1+	28	24.7	161 - 170	VLQPSSVDSQ
515.2	515.3	1+	18	27.3	174 - 178	VLVNA
953.5	953.4	1+	18	21.5	189 - 196	FKDEDTQA
879.5	879.4	1+	9	35.2	197 - 203	MPFRVTE
1007.6	1007.5	1+	11	33.7	197 - 204	MPFRVTEQ
818.5	818.4	1+	21	25.0	205 - 211	ESKPVQM
761.5	761.5	1+	21	31.9	227 - 232	KMKILE
579.4	579.3	1+	16	23.1	234 - 239	PFASGT
710.4	710.3	1+	11	30.8	234 - 240	PFASGTM
716.4	716.3	1+	24	31.3	247 - 253	PDEVSGL
722.5	722.4	1+	10	42.5	257 - 262	ESIINF
901.7	901.5	1+	18	25.4	275 - 281	EERKIKV
1048.7	1048.7	1+	11	36.2	276 - 283	ERKIKVYL
662.5	662.3	1+	8	25.9	284 - 288	PRMKM
875.5	875.5	1+	8	27.1	319 - 326	AESLKISQ
1132.7	1132.6	1+	11	23.4	323 - 333	KISQAVHAAHA
676.4	676.4	1+	41	18.1	327 - 333	AVHAAHA
805.6	805.4	1+	11	18.1	327 - 334	AVHAAHAE
918.5	918.5	1+	20	26.8	327 - 335	AVHAAHAEI
1161.7	1161.6	1+	21	23.8	327 - 337	AVHAAHAEINE
687.3	687.4	1+	24	21.5	338 - 344	AGREVVG
845.5	845.4	1+	17	22.3	338 - 346	AGREVVGSA
889.5	889.4	1+	11	33.8	359 - 365	FRADHPF
855.5	855.4	1+	16	36.2	360 - 366	RADHPFL
682.5	682.4	1+	14	22.9	369 - 374	IKHIAT
796.5	796.5	1+	17	21.0	369 - 375	IKHIATN
867.6	867.5	1+	19	22.8	369 - 376	IKHIATNA

Annexe IV. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 3 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP3).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
783.4	1565.8	2+	9	28.6	42 - 55	VYLGAKDSTRTQIN
847.5	1693.9	2+	12	27.1	42 - 56	VYLGAKDSTRTQINK
676.4	676.4	1+	28	37.2	61 - 66	DKLPGF
935.5	935.4	1+	11	34.1	61 - 69	DKLPGFGDS
1028.4	1028.5	1+	7	25.3	89 - 97	NQITKPNDV
949.5	949.5	1+	26	27.8	91 - 98	ITKPNDVY
1036.6	1036.5	1+	13	25.9	91 - 99	ITKPNDVYS
646.4	646.4	1+	8	32.5	101 - 106	SLASRL
790.5	790.4	1+	19	38.7	110 - 115	ERYPIL
807.4	807.4	1+	6	38.1	125 - 131	LYRGGLE
1150.1	2299.2	2+	6	31.4	142 - 161	ARELINSWVESQTNGIIRNV
1225.8	1225.7	1+	8	36.9	157 - 167	IIRNVLQPSSV
879.5	879.4	1+	15	35.2	197 - 203	MPFRVTE
1007.6	1007.5	1+	23	33.7	197 - 204	MPFRVTEQ
748.4	748.4	1+	12	29.8	198 - 203	PFRVTE
876.5	876.5	1+	8	33.6	198 - 204	PFRVTEQ
761.5	761.5	1+	15	31.9	227 - 232	KMKILE
928.5	928.4	1+	18	36.6	234 - 242	PFASGTMSM
829.2	829.4	1+	14	37.0	246 - 253	LPDEVSGL
722.4	722.4	1+	18	42.4	257 - 262	ESIINF
1048.8	1048.7	1+	7	36.1	276 - 283	ERKIKVYL
919.7	919.6	1+	11	35.7	277 - 283	RKIKVYL
1132.8	1132.6	1+	20	23.6	323 - 333	KISQAVHAAHA
1002.6	1002.5	1+	7	39.6	359 - 366	FRADHPFL
855.5	855.4	1+	25	36.2	360 - 366	RADHPFL

Annexe V. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 4 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP4).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
903.5	1805.9	2+	9	35.6	28 - 43	IFYCPIAIMSALAMVY
783.4	1565.8	2+	21	28.6	42 - 55	VYLGAKDSTRTQIN
676.4	676.4	1+	15	37.1	61 - 66	DKLPGF
848.5	848.4	1+	16	34.5	61 - 68	DKLPGFGD
949.6	949.5	1+	11	27.8	91 - 98	ITKPNDVY
632.5	632.4	1+	8	34.2	227 - 231	KMKIL
874.6	874.5	1+	21	41.5	227 - 233	KMKILEL
746.6	746.4	1+	23	43.3	228 - 233	MKILEL
556.3	556.3	1+	16	34.1	244 - 248	VLLPD
1177.9	1177.7	1+	24	36.3	275 - 283	EERKIKVYL
647.5	647.4	1+	9	29.9	281 - 285	VYLPR
855.6	855.4	1+	9	36.1	360 - 366	RADHPFL
699.5	699.3	1+	8	37.3	361 - 366	ADHPFL

Annexe VI. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 5 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP5).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1210.8	1210.6	1+	8	25.7	42 - 52	VYLGAKDSTRT
783.5	1565.8	2+	15	28.6	42 - 55	VYLGAKDSTRTQIN
676.4	676.4	1+	31	37.1	61 - 66	DKLPGF
848.4	848.4	1+	18	34.4	61 - 68	DKLPGFGD
755.6	755.4	1+	23	28.3	78 - 84	VNVHSSL
949.6	949.5	1+	20	27.8	91 - 98	ITKPNDVY
1036.6	1036.5	1+	17	25.8	91 - 99	ITKPNDVYS
790.5	790.4	1+	17	38.6	110 - 115	ERYPIL
791.5	2372.2	3+	7	78.0	150 - 171	VESQTNGIIRNVLQPSSVDSQT
727.6	727.5	1+	16	35.8	157 - 162	IIRNVL
1225.8	1225.7	1+	11	36.8	157 - 167	IIRNVLQPSSV
879.6	879.4	1+	9	35.2	197 - 203	MPFRVTE
710.4	710.4	1+	8	34.1	218 - 223	FRVASM
761.5	761.5	1+	24	31.9	227 - 232	KMKILE
615.5	615.4	1+	11	39.2	229 - 233	KILEL
823.4	823.4	1+	14	39.3	233 - 240	LPFASGTM
710.3	710.3	1+	22	30.7	234 - 240	PFASGTM
829.5	829.4	1+	8	36.9	246 - 253	LPDEVSGL
722.5	722.4	1+	25	42.4	257 - 262	ESIINF
919.8	919.6	1+	16	35.5	277 - 283	RKIKVYL
1132.8	1132.6	1+	33	23.5	323 - 333	KISQAVHAAHA
1161.7	1161.6	1+	11	23.9	327 - 337	AVHAAHAEINE
1002.7	1002.5	1+	11	39.5	359 - 366	FRADHPFL
855.6	855.4	1+	16	36.0	360 - 366	RADHPFL

Annexe VII. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 1 of the hydrolysate of ovalbumin with pepsin separated by anion exchange chromatography (AE-OP1).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1204.8	1204.6	1+	26	19.9	17 - 26	KELKVHHANE
716.4	1431.8	2+	24	24.9	17 - 28	KELKVHHANENI
947.7	947.5	1+	13	19.6	19 - 26	LKVHHANE
834.5	834.4	1+	21	15.6	20 - 26	KVHHANE
706.4	706.3	1+	24	15.0	21 - 26	VHHANE
522.3	522.3	1+	20	29.4	42 - 46	VYLGA
765.4	765.4	1+	17	25.3	42 - 48	VYLGAKD
783.4	1565.8	2+	22	27.8	42 - 55	VYLGAKDSTRTQIN
652.4	1303.7	2+	9	22.6	44 - 55	LGAKDSTRTQIN
716.5	1431.8	2+	7	21.4	44 - 56	LGAKDSTRTQINK
835.4	835.4	1+	17	14.7	45 - 52	GAKDSTRT
934.5	934.5	1+	13	19.4	48 - 55	DSTRTQIN
676.5	676.4	1+	29	36.4	61 - 66	DKLPGF
755.5	755.4	1+	26	27.4	78 - 84	VNVHSSL
656.4	656.3	1+	22	23.8	79 - 84	NVHSSL
929.5	929.5	1+	22	20.2	89 - 96	NQITKPND
1028.6	1028.5	1+	19	24.7	89 - 97	NQITKPNDV
687.4	687.4	1+	23	19.0	91 - 96	ITKPND
786.5	786.4	1+	14	23.6	91 - 97	ITKPNDV
949.5	949.5	1+	29	27.0	91 - 98	ITKPNDVY
1036.6	1036.5	1+	16	25.1	91 - 99	ITKPNDVYS
646.5	646.4	1+	7	31.7	101 - 106	SLASRL
790.5	790.4	1+	15	37.8	110 - 115	ERYPIL
661.5	661.4	1+	8	37.6	111 - 115	RYPIL
1165.7	1165.6	1+	7	41.9	126 - 135	YRGGLEPINF
616.5	616.3	1+	9	43.6	141 - 145	QAREL
727.5	727.5	1+	10	34.6	157 - 162	IIRNVL
630.4	630.3	1+	21	21.0	161 - 166	VLQPSS
729.5	729.4	1+	23	28.0	161 - 167	VLQPSSV
879.5	879.4	1+	15	34.3	197 - 203	MPFRVTE
1007.6	1007.5	1+	12	32.7	197 - 204	MPFRVTEQ
687.4	687.4	1+	22	17.0	205 - 210	ESKPVQ
818.5	818.4	1+	11	24.1	205 - 211	ESKPVQM
949.5	949.4	1+	24	29.6	205 - 212	ESKPVQMM
593.3	593.3	1+	24	38.0	213 - 217	YQIGL
710.4	710.4	1+	8	32.9	218 - 223	FRVASM
563.3	563.3	1+	15	22.3	219 - 223	RVASM
806.5	806.4	1+	13	26.7	224 - 230	ASEKMKI
1048.7	1048.6	1+	21	31.9	224 - 232	ASEKMKILE
761.5	761.5	1+	22	30.8	227 - 232	KMKILE
874.6	874.5	1+	15	41.4	227 - 233	KMKILEL
692.4	692.4	1+	8	33.4	233 - 239	LPFASGT
823.5	823.4	1+	12	39.2	233 - 240	LPFASGTM
910.5	910.4	1+	24	37.0	233 - 241	LPFASGTMS
579.3	579.3	1+	8	22.7	234 - 239	PFASGT
710.4	710.3	1+	22	30.1	234 - 240	PFASGTM
797.4	797.3	1+	19	27.4	234 - 241	PFASGTMS
722.4	722.4	1+	11	42.4	257 - 262	ESIINF
593.3	593.3	1+	12	42.1	258 - 262	SIINF
594.3	594.3	1+	17	18.7	268 - 272	WTSSN
1177.8	1177.7	1+	8	35.4	275 - 283	EERKIKVYL
673.5	673.4	1+	16	17.2	276 - 280	ERKIK
1048.8	1048.7	1+	9	34.8	276 - 283	ERKIKVYL
662.4	662.3	1+	10	24.7	284 - 288	PRMKM
725.4	725.3	1+	9	28.9	307 - 313	FSSSANL

1132.8	1132.6	1+	22	22.9	323 - 333	KISQAVHAAHA
891.5	891.4	1+	16	22.8	325 - 333	SQAVHAAHA
605.4	605.3	1+	17	15.7	327 - 332	AVHAAH
676.4	676.4	1+	42	17.7	327 - 333	AVHAAHA
605.4	605.3	1+	21	17.2	328 - 333	VHAAHA
687.4	687.4	1+	22	21.0	338 - 344	AGREVVG
845.5	845.4	1+	22	21.9	338 - 346	AGREVVGSA
1002.6	1002.5	1+	24	38.7	359 - 366	FRADHPFL
855.5	855.4	1+	11	34.1	360 - 366	RADHPFL
682.5	682.4	1+	21	22.4	369 - 374	IKHIAT
796.5	796.5	1+	16	20.5	369 - 375	IKHIATN
867.6	867.5	1+	13	22.5	369 - 376	IKHIATNA

Annexe VIII. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 2 of the hydrolysate of ovalbumin with pepsin separated by anion exchange chromatography (AE-OP2).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
508.2	508.2	1+	15	17.7	6, - 10	AASME
753.5	753.5	1+	18	20.5	17 - 22	KELKVH
1204.7	1204.6	1+	34	20.3	17 - 26	KELKVHHANE
789.9	1578.8	2+	8	26.5	17 - 29	KELKVHHANENIF
706.4	706.3	1+	13	15.1	21 - 26	VHHANE
522.2	522.3	1+	9	29.7	42 - 46	VYLGA
765.5	765.4	1+	15	25.8	42 - 48	VYLGAKD
953.6	953.5	1+	30	25.7	42 - 50	VYLGAKDST
848.5	848.4	1+	24	34.0	61 - 68	DKLPGFGD
935.6	935.4	1+	14	33.7	61 - 69	DKLPGFGDS
827.5	827.4	1+	8	23.5	81 - 87	HSSLRDI
687.4	687.4	1+	15	19.3	91 - 96	ITKPND
949.6	949.5	1+	9	27.4	91 - 98	ITKPNDVY
1036.6	1036.5	1+	6	25.5	91 - 99	ITKPNDVYS
919.6	919.5	1+	15	38.8	109 - 115	EERYPIL
790.5	790.4	1+	14	38.2	110 - 115	ERYPIL
807.5	807.4	1+	13	28.3	125 - 131	LYRGGLE
694.4	694.4	1+	12	24.3	126 - 131	YRGGLE
734.4	734.3	1+	17	13.6	150 - 156	VESQTNG
844.5	844.4	1+	19	26.2	161 - 168	VLQPSSVD
931.6	931.5	1+	18	25.7	161 - 169	VLQPSSVDS
517.4	517.3	1+	10	16.6	163 - 167	QPSSV
620.3	620.3	1+	11	15.5	167 - 172	VDSQTA
653.4	653.3	1+	18	19.1	189 - 193	FKDED
754.4	754.3	1+	14	19.9	189 - 194	FKDEDT
882.5	882.4	1+	10	19.6	189 - 195	FKDEDTQ
953.5	953.4	1+	18	21.2	189 - 196	FKDEDTQA
879.5	879.4	1+	8	34.7	197 - 203	MPFRVTE
593.4	593.3	1+	26	38.1	213 - 217	YQIGL
740.5	740.4	1+	13	45.6	213 - 218	YQIGLF
1065.6	1065.5	1+	13	45.4	231 - 240	LELPFASGTM
579.3	579.3	1+	29	23.0	234 - 239	PFASGT
710.3	710.3	1+	23	30.4	234 - 240	PFASGTM
928.5	928.4	1+	8	36.3	234 - 242	PFASGTMSM
798.4	798.5	1+	12	40.7	243 - 249	LVLLPDE
685.4	685.4	1+	40	33.5	244 - 249	VLLPDE
1041.7	1041.6	1+	15	41.8	244 - 253	VLLPDEVSGL
829.5	829.4	1+	17	36.8	246 - 253	LPDEVSGL
504.1	504.3	1+	7	16.0	250 - 254	VSGLE
718.5	718.4	1+	6	28.7	254 - 259	EQLESI
575.3	575.3	1+	13	26.8	257 - 261	ESIIN
722.4	722.4	1+	19	42.3	257 - 262	ESIINF
763.5	763.4	1+	9	37.0	260 - 265	INFEKL
619.4	619.3	1+	10	20.7	263 - 267	EKLTE
824.4	824.3	1+	9	23.3	266 - 272	TEWTSSN
594.3	594.3	1+	23	18.9	268 - 272	WTSSN
706.4	706.3	1+	15	34.9	300 - 306	AMGITDV
536.1	536.2	1+	20	25.9	301 - 305	MGITD
635.4	635.3	1+	21	34.3	301 - 306	MGITDV
504.2	504.3	1+	23	27.7	302 - 306	GITDV
651.3	651.3	1+	15	40.6	302 - 307	GITDVF
468.2	468.3	1+	15	17.0	327 - 331	AVHAA
605.4	605.3	1+	20	15.7	327 - 332	AVHAAH
676.4	676.4	1+	18	18.1	327 - 333	AVHAAHA
805.5	805.4	1+	35	17.9	327 - 334	AVHAAHAE

918.6	918.5	1+	26	26.4	327 - 335	AVHAAHAEI
1032.6	1032.5	1+	27	22.7	327 - 336	AVHAAHAEIN
1161.7	1161.6	1+	31	23.6	327 - 337	AVHAAHAEINE
854.4	854.4	1+	14	21.7	330 - 337	AAHAEINE
974.6	974.5	1+	7	22.1	338 - 347	AGREVVGSAE
561.3	561.3	1+	9	18.1	342 - 347	VVGSAE
692.3	692.3	1+	9	18.4	352 - 358	AASVSEE
1131.6	1131.6	1+	11	39.8	358 - 366	EFRADHPFL
855.5	855.4	1+	25	35.4	360 - 366	RADHPFL